INTRINSIC HEALING IN THE EQUINE SUPERFICIAL DIGITAL FLEXOR TENDON IN VITRO: EFFECTS OF HYALURONATE AND POLYSULFATED GLYCOSAMINOGLYCANS ON MATRIX SYNTHESIS AND CELL PROLIFERATION

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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ABSTRACT

The use of representative in vitro models has been favoured for the study of biologic and pharmacologic responses of the equine superficial digital flexor tendon (SDFT). The objectives of this study were to determine the effects of exogenous sodium hyaluronate (NaHA) and polysulfated glycosaminoglycan (PSGAG) on extracellular matrix (ECM) synthesis and cell proliferation by explants of the equine SDFT, and to compare the effects of NaHA and PSGAG at equivalent concentrations. Explants of the tensile SDFT were cultured in RPMI 1640/10% DHS/100 μg/ml ascorbate in sealed bottles on rollers (14 rpm) at 36.5±0.5°C. After a pre-treatment culture period of 18 days, cultures were randomized into treatment groups, and the drug being tested added to the medium. Cultures were treated for 6 days prior to pulse-chase radiolabeling with ^{35}S-sulfate, L-[2,3,4,5-^{3}H] proline and methyl-C^{14}-thymidine to determine rates of glycosaminoglycans (GAGs) synthesis, protein synthesis and cell proliferation respectively. The explants and aliquots of medium were hydrolysed in 6 M HCl, scintillation counted, and explant and medium counts summed in order to determine molar incorporation rates per mg dry weight of tissue. Protein in aliquots of the medium was precipitated and hydrolysed. The rates of collagen and noncollagen protein synthesis were determined from the derivatized amino acids by RP-HPLC. Hyaluronate synthesis was determined by radiometric assay in one experiment.

NaHA was evaluated at concentrations of 0 to 2000 μg/ml. The rates of proline incorporation into protein, collagen synthesis and GAGs synthesis were increased in a dose-dependent manner. Neither noncollagen protein synthesis nor cell proliferation were significantly affected by NaHA. PSGAG was added to the medium at concentrations of 0 to 5000 μg/ml. The rates of proline incorporation into protein, collagen and noncollagen protein synthesis, and GAGs synthesis were significantly increased in the presence at concentrations higher than 100 μg/ml. The degree of response to PSGAG is strongly dose-related, with optimal concentrations for increased synthesis occurring at 1000 to 2000 μg/ml PSGAG. The effects of NaHA were compared to those of PSGAG, both at 1000 μg/m. The exogenous administration of PSGAG results in a wider range and greater degree of cellular

responses than NaHA. Exogenous PSGAG resulted in increased collagen, noncollagen protein, and sulfated GAG synthesis, increased cell proliferation, and decreased hyaluronate synthesis. However, exogenously administered NaHA only increased collagen and sulfated GAG synthesis and the degree of response was significantly less than the response to PSGAG. This data provides empirical evidence that substantiates and identifies a cellular basis for the beneficial effects of NaHA and PSGAG on equine tendon repair. The results suggest that PSGAG may be a more potent drug for the stimulation or modulation of cell proliferation and ECM in the injured equine SDFT.

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In memory of my grandparents Eskha and Ivy Halls

and Christine and Stan Wolfe

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ßAPN	beta-aminoproprionitrile
BrdU	5-bromo-2'deoxyuridine
CS	chondroitin sulfate
CtS	citrate synthestase
DDFT	deep digital flexor tendon
DHS	donor horse serum
DOCA	7-deoxycholic acid
D-PEN	D-penicillinamine
DS	dermatan sulfate
ECM	extracellular matrix
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GDH	glutamate dehydrogenase
Gly	glycine
H	heparin
HA	hyaluronate
HMW	high molecular weight
HS	heparin sulfate
Hyp	hydroxyproline
IGF-I	insulin-like growth factor I
KS	keratan sulfate
LMW	low molecular weight
LP	link protein

MDH	maleate dehydrogenase
MW, *mw*	molecular weight
MMP	matrix metalloproteinase
NaHA	sodium hyaluronate; Hyonate®
NSAID	non-steroidal anti-inflammatory drug
OPA	*o*-phtalaldehyde
PG	proteoglycan
Pro	proline
PSGAG	polysulfated glycosaminoglycans; Adequan®
rhIGF-I	recombinant human insulin-like growth factor I
RP-HPLC	reverse phase high performance liquid chromatography
SDFT	superficial digital flexor tendon
SPA	specific activity
TCA	trichloracetic acid
TIMP	tissue inhibitor of metalloproteinase

CHAPTER ONE

1.0 GENERAL INTRODUCTION

The tendons and ligaments of the distal equine limbs are dynamic structures capable of withstanding high loads and strains (Denoix 1996). They provide support to the fetlock, prevent carpal hyperextension, and transmit the energy of impact and full weight bearing during propulsion and limb lift off. Injuries affecting equine tendons and ligaments result in significant mortality, morbidity and financial loss in the performance horse industries (Wilson *et al* 1996; Wilson and Robinson 1996). The superficial digital flexor tendon (SDFT) of the forelimb is the most commonly injured tendon and although lacerations are common, the most frequently reported lesions are related to acute exacerbation of degenerative tendinitis (Foland *et al* 1991; Belknap *et al* 1993; Wilson *et al* 1996). Recent estimates of the rate of occurrence of SDFT injury range from 10 to 30% of horses in training or racing in Europe and North America depending upon breed and activity, with 4.3 to 11.3% of racing fatalities attributed to tendon rupture or damage (Rossdale *et al* 1985; Anonymous 1991; McKee and Clarke 1993; Goodship *et al* 1994; Peloso *et al* 1994; Estberg *et al* 1996). Of 1036 equine racing injuries resulting in death in the United Kingdom, horses competing in the steeple-chase had the greatest occupational risk of death following tendon rupture or damage (11.3%), followed by hurdles (9.4%), National Hunt flat racers (9.1%) and flat racers (4.3%) (McKee and Clarke 1993). In studies of injuries at American racetracks, the incidence of tendon injury in Thoroughbreds was greater than in Standardbreds or Quarter horses (Wilson *et al* 1996). In addition to the high rate of occurrence the prognosis for complete recovery to the previous level of performance is often poor, horses that return to high performance activity following injury and reparative scar formation are more likely to become lame with moderate work, and re-injury is common (Rooney and Genovese 1981; Silver *et al* 1983;

Mohammed *et al* 1991; Mohammed *et al* 1992; Marr *et al* 1993; Peloso *et al* 1994; Wilson *et al* 1996; Wilson and Robinson 1996).

Although there have been many histologic studies investigating the cellular nature of equine tendon lesions, relatively few studies have investigated the biologically active factors involved in tendon repair and their possible manipulation in promoting restoration of the physical integrity and tensile strength of the original tendon structure (Stromberg 1971; Webbon 1977; Williams *et al* 1980). Recent *in vitro* studies have started to address this deficit in current knowledge and answer questions pertaining to the biological and biochemical aspects of tendon physiology and healing (Riley *et al* 1996; Dahlgren *et al* 1997; Murphy and Nixon 1997).

There has been considerable controversy in the literature over the relative importance of the intra-tendinous (intrinsic) tissue versus the peri-tendinous (extrinsic) tissues to the blood supply and cellular contributions to tendon healing, particularly for centrally located 'core' lesions in which there is little or no disruption of the epitendon or paratenon. However, recent studies in many species, including the horse, support the development of treatment methods aimed at enhancement of intrinsic contributions to tendon healing (Graham *et al* 1981; Manske *et al* 1984; Lunborg and Rank 1987; Mass and Tuel 1991; Kraus-Hansen *et al* 1992, Riley *et al* 1996; Murphy and Nixon 1997). Fibroblast growth factor, platelet derived growth factor and insulin-like growth factor-I have been demonstrated to have potent stimulatory effects on protein synthesis, proteoglycan synthesis, and cell proliferation in avian, equine, rat and rabbit tendon cultures (Gauger *et al* 1985; Stein 1985, Abrahamsson *et al* 1991a&b; Murphy and Nixon 1997). The results of these studies suggest that exogenous growth factors and other biologically active molecules may be of importance in intrinsic tendon healing, and useful for the treatment of tendons *in vivo*.

Pharmacological agents have been used experimentally in man and animals during the repair phase of tendon healing but have failed to consistently reduce adhesion formation, promote more rapid tendon healing or reduce scar formation in clinical cases of tendon disease (Gaughan *et al* 1991). The administration of exogenous polysulphated glycosaminoglycans, hyaluronate and beta-aminopropionitrile fumarate have recently been

investigated *in vivo* in clinical trials and studies (Churchill 1985; Gaughan *et al* 1991; Foland *et al* 1992; Gift *et al* 1992; Redding *et al* 1992; Marr *et al* 1993; Gaughan *et al* 1995; Genovese *et al* 1996; Reef *et al* 1996). The results of these trials have demonstrated some support for the use of these drugs but the studies have generally had poor statistical power, variable and/or equivocal results, or have used a model for tendon injury which has questionable relevance or suitability for determination of lesion responses to pharmaceutical agents. Responses to the drugs in the studies were largely determined by indirect assessment methods such as ultrasonography or return to performance activity, and the studies did not provide information on the cellular or molecular responses of tendon tissue to these drugs (Marr *et al* 1993; Gaughan *et al* 1995; Genovese *et al* 1996; Reef *et al* 1996).

In order to achieve an understanding of the biological or biochemical responses of tendon at the cellular and molecular level to cytokines and drugs, representative *in vitro* tissue culture models have been highly valued as tools for studying the repair phase of tendon healing (Graham *et al* 1981; Gelberman *et al* 1984; Manske *et al* 1984; Mass and Tuel 1991).

The objectives of the present study were to investigate the intrinsic synthetic metabolic responses of the equine SDFT to commercial preparations of polysulphated glycosaminoglycans and hyaluronate using a modification of a previously developed *in vitro* submerged explant tissue culture model of the equine forelimb SDFT (Riley *et al* 1996). This approach has been supported in recent literature as a suitable technique for investigating the effects of pharmaceutical agents and growth factors on the equine SDFT (Riley *et al* 1996; Dahlgren *et al* 1997; Murphy and Nixon 1997).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Normal Tendon Development

There has been little definitive work investigating the developmental changes that occur specifically in the equine SDFT, but considerable insight into tendon development has been obtained from studies other tendons, and tendons and ligaments in other species (Otomo 1973). During limb bud formation in the rat embryo, histologic changes occur which are characterized by changes in cell morphology and density with respect to the extracellular matrix (ECM). Initially, undifferentiated mesenchymal cells coalesce in the regions adjacent to the primordial digit (Han and Cormack 1979). As the mesenchymal skeleton differentiates into cartilage, dense bundles of fibroblasts differentiate into numerous longitudinally arranged tendon cells with plump granular nuclei, between which are eosinophilic collagen fibres (Otomo 1973; Webbon 1978; Ham and Cormack 1979). As development progresses in the newborn animal there is a decrease in cellular density and a concomitant increase in the fibril component and collagen concentration of the ECM (Webbon 1977; Webbon 1978; Vailas *et al* 1985). Shortly after birth the epitendon has a histologic appearance that is distinct from the tendon tissue which it surrounds (Otomo 1973). In young horses the tendon cells are numerous with plump slightly elongated nuclei which tend to become further elongated in older horses. Over the age of 5 years, acellular areas develop in the metacarpal regions (Webbon 1978). Chondroid metaplasia also tends to be seen most frequently in older horses (Smith and Webbon 1996).

Accompanying these cellular changes are changes in the quantity and quality of the extracellular matrix, a composite of two main classes of molecules: (1) polysaccharide chains

referred to as glycosaminoglycans (GAGs), which (with the exception of hyaluronate) are covalently linked to protein in the form of proteoglycans (PGs), and (2) fibrous proteins which are either structural (eg. collagen and elastin) or adhesive (eg. fibronectin and laminin) in nature. Of the matrix fibrous proteins, the most ubiquitous and widely studied type is collagen (Bailey and Light 1989). The rate of synthesis and therefore the relative amounts of collagen present increase change embryonic development, as do the physical characteristics and distribution of the collagen fibrils (Diegelmann and Peterkofsky 1972; Parry and Craig 1988; Birk and Zycband 1994). The fetal or immature connective tissues initially have widely spread bundles of small, relatively constant diameter fibrils, oriented in a random manner (Parry and Craig 1988). In precocial animals the distribution of collagen fibril diameters broadens considerably before birth, parallelling the requirements for the resistance of tensile force in the limb. In most species the mean fibril diameter increases substantially, and assumes a unimodal or bimodal (as is the case for the adult horse) distribution at maturity (Parry *et al* 1978; Craig and Parry 1988; Birk and Zycband 1994). After maturation, mean fibril diameter decreases with advancing age, a change that has been reported to be more distinct in the horse than in other species (Parry *et al* 1978). More definitive work examining fibril diameter in the equine SDFT has recently demonstrated that mean fibril diameter doubles within the first 5 years of life compared to fetal diameters, plateaus in the five to ten year old age group, and tends to decline in older horses (Patterson-Kane *et al* 1997a). These workers concluded that the rapid rate at which this occurs in the SDFT of athletic horses may be a pathological response induced by training for performance activity (Patterson-Kane *et al* 1997b). Further research is required to verify this hypothesis.

Investigations into developmental aspects of the PGs or GAG components of the tendon ECM have been limited. In general, all fetal connective tissues are rich in hyaluronate (HA), a ubiquitous GAG which plays an essential role in cell proliferation, migration and differentiation (McCallion and Ferguson 1996). Examination of its role in other fetal tissues has determined that its action regulated mainly via the CD44 and RHAMM transmembrane molecules, the principal cell surface receptors for HA (Knudson 1993; Sherman *et al* 1994; McCallion and Ferguson 1996). Hyaluronate is also able to inhibit cell differentiation and

bind growth factors, thus influencing cell growth and differentiation by changing the local concentrations of these factors (Kujawa *et al* 1986; Ruoslahti and Yamaguchi 1991). Therefore HA appears to play an especially important role the highly cellular phase of connective tissue development (including tendon formation). Relationships between fetal hyaluronate levels and angiogenesis, collagen synthesis and fibril development have also been identified (McCallion and Ferguson 1996). Degradation products of HA may stimulate fetal angiogenesis and increase type I collagen synthesis which is though to provide a substrate for endothelial cell migration in the fetus (Kumar *et al* 1992). High concentrations of HA and PGs in early fetal development of the chick, rabbit, rat and bovine tendons are associated with thin fibrils which increase in diameter only after these concentrations drop to low levels (Scott 1980; Scott and Hughes 1986; Scott 1988; Scott 1990).

Although interaction between PGs and collagen have been documented in fetal and adult tissues, there are few studies which have examined changes in the concentration and types of PGs during fetal development (McCallion and Ferguson 1996). It has been determined that as tendon matures, and large collagen fibrils form, the PGs molecules that link as many as four collagen fibrils in the fetal and neonatal tendon may be trapped in the coalescing fibrillar mass (Scott 1990). Conversely, PGs may also inhibit fibril formation. For example, the small dermatan sulfate (DS) containing PG has been demonstrated to inhibit fibril formation of types I and II collagen in bovine tendon *in vitro* (Vogel *et al* 1984). Differences in the PGs content of fetal and adult tendon have been demonstrated in the rat and bovine. In the rat, chondroitin sulfate (CS) and HA were demonstrated as the major GAGs in foetal tendon, whereas DS became the predominant GAG within several weeks after birth (Scott *et al* 1981). In fetal bovine tendon, smaller PGs have been found to predominate, whereas the large PGs found in the compressive regions (reflecting a change in function) of adult tendons were not found (Vogel and Evanko 1985; Vogel and Heinegard 1987). Recently Smith and Webbon (1996) reported a change in the distribution of keratan sulfate (KS) in the flexor tendons when foals were compared to adult horses. There was minimal staining for KS at all levels of the foal flexor tendons (although there was increased intensity in the metacarpophalangeal regions). Staining for KS became more marked in the

metacarpophalangeal and phalangeal regions with increasing age, but was low in the metacarpal region (Smith and Webbon 1996). Although differences have been detected between fetal and adult tendons which are believed to reflect functional requirements, their importance to the development of the tendon has not been clearly elucidated, and further study of the role of PGs is required (Vogel and Heinegard 1987; Jones and Bee 1990).

2.2 Histologic Structure

There have been few studies investigating the histologic and ultrastructural nature of normal equine tendons (Webbon 1978). The structural properties of mammalian tendons are complex, with unique morphological characteristics (Wilmink *et al* 1992). Longitudinal sections of the SDFT reveal distinct patterns of longitudinal parallel collagen fibres in bundles between which are elongated tendon cells which are often arranged in rows (Norberg *et al* 1967; Stromberg 1971). The basic tendon unit is called the fibril which consists of an ordered arrangement of microfibrils. These have transverse bands with a characteristic periodicity of approximately 70 nm which disappear when tension is applied to the fibrils, and reappear on relaxation (Evans and Barbenel 1975). Aggregations of the fibrils form collagen fibres which occur in a planar waveform or 'crimp' pattern that appears as dark and light transverse banding under polarized light (Wilmink *et al* 1992; Patterson Kane *et al* 1997a). Aggregations of these extracellular protein threads (collagen fibres) and associated tendon cells with their processes, form primary tendon bundles (subfasicular units) which have a diameter up to 300 μm. The primary bundles are arranged into larger secondary units (fascicles) with a diameter of 600 μm in an open helical pattern which form into more complex tertiary bundles (Webbon 1973; Evans and Barbenel 1975). The tertiary bundles are bordered on either side by a thin layer of endotendon (Stromberg 1971). Transverse sections of tendon reveal that the endotendon regions between the tendon bundles usually contain an artery accompanied by two veins (Stromberg 1971).

2.3 Nutrient Pathways

There has been considerable controversy regarding the role of perfusion versus diffusion for the supply of nutrients and oxygen, and the removal of metabolic and gaseous wastes from the equine SDFT in health and disease (Smith and Webbon 1996). Although tendon tissue has been demonstrated to be more tolerant of hypoxic conditions *in vitro* than other tissues, hypoxia has been implicated in the development of tendinitis or degenerative tendinopathy (Józsa *et al* 1982; Webster and Burry 1982; Birch 1993). Ligation of the intratendinous blood supply of the mid-metacarpal region of the equine SDFT has produced lesions consistent with those documented in cases of naturally occurring tendinitis but further study with sham controls is required to validate this protocol as a model for tendon injury (Kraus-Hansen *et al* 1992). These results concurred with an earlier study in the rabbit in which an intact blood supply was determined to be essential to tendon remodelling (Landi *et al* 1980b). Therefore the vascular and microvascular anatomy of the equine SDFT has been considered to be of particular importance with respect to the aetiology and pathogenesis of tendon disease (Stromberg and Tufvesson 1969; Webbon 1973; Kraus-Hansen *et al* 1992).

In the tensile portion of the SDFT, the intratendinous blood supply is composed of two major parallel vessels which course distally in the medial and lateral borders with an extensive interlacing network of perpendicularly arranged intratendinous arterioles with accompanying small venules and finer longitudinally arranged vessels which course between the collagen bundles (Norberg *et al.* 1967; Stromberg 1969; Stromberg 1971; Kraus-Hansen *et al* 1992). Several reports have indicated that the intratendinous blood supply is poorer in the mid-metacarpal segment of the SDFT, giving rise to an anatomical 'watershed zone' into which nutrients must diffuse, and which many authors believe may predispose this area to vascular injury and the development of tendinitis (Stromberg and Tufvesson 1969; Webbon 1973; Kraus-Hansen *et al* 1992). Stromberg (1971) concluded that metabolic stimulation, including exercise, only slightly enhanced intratendinous capillary flow in horses. In rabbits exercise resulted in increased blood flows of approximately 140% above resting values

(Landi *et al* 1983). Following temporary occlusion of the peripheral circulation of the distal equine forlimb (using a tourniquet applied immediately above the carpus), maximal blood flows of only 60.2±70.2% above resting values were achieved (Stromberg 1971). It has been suggested but not substantiated, that stretching the tendon during physical activity may result in increased diffusion out of the tendon, instead of an increased flow to accommodate metabolic demand (Birch 1993).

In human flexor tendons, diffusion is considered to be an important pathway for supplying the tissue with nutrients particularly in regions where the tendon is surrounded by a synovial sheath (Manske and Lesker 1987). In avian studies in which the flexor tendon blood supply was severed, tritiated proline uptake was comparable to that of tendon with an intact blood supply (Manske *et al* 1978a). Other studies have found that when intra synovial rabbit tendon segments have their blood supply interrupted or are placed into a synovial environment they remain viable, indicating that they may be nourished by the synovial fluid environment (Lunborg and Rank 1987; Manske and Lesker 1987; Weber 1987). In the equine SDFT, those regions surrounded by synovial fluid are limited to the digital sheath and the carpal sheath. Studies have not been published to date which have demonstrated the contribution of diffusion in these areas to nutrition of either the SDFT or deep digital flexor tendon (DDFT), and these regions are not commonly affected by tendon injury. However, explants of the equine SDFT of up to 5 mm thickness x 4 mm diameter have been cultured *in vitro*, without evidence of degeneration in the centre of the explants, or loss of the ability to synthesize molecules of the ECM, confirming the plausibility of diffusion as a possible nutrient pathway in the tensile equine SDFT (Riley *et al* 1996).

Currently, both diffusion and perfusion are supported as routes for the nutrition of the flexor tendons in man, monkeys, dogs, rabbits and chickens, particularly for intra synovial tendons (Manske *et al* 1978a; Manske *et al* 1978b; Mankse and Lesker 1982). However, studies comparing the two routes have concluded that diffusion is the more important of the pathways (Manske and Lesker 1987; Weber 1987). It has been speculated that diffusion may play an important role in the nutrition of equine tendons, but similar studies comparing perfusion versus diffusion have not been performed in the horse (Smith and Webbon 1996).

2.4 The Cellular Components of Tendons

2.4.1 Cell Morphology and Function

For over 60 years it has been recognized that each subpopulation of fibroblast-like cells has unique properties related to the tissue of origin of those cells (Parker 1933). The morphological appearance of the tenocyte population appears to be variable, yet little has been done to characterize the properties of different cell populations within equine tendon (Banes *et al* 1988).

Equine tenocytes have been arbitrarily classified on the basis of histologic characteristics alone into three morphologically distinct subpopulations: type I (cells with thin spindle-shaped nuclei), type II (linearly arranged groups of cells with more rounded, cigar shaped nuclei) and type III (cartilage-like cells with round nuclei and visible nucleoli) cells (Webbon 1978; Webster and Burry 1982; Riederer-Henderson 1983; Goodship and Birch 1996; Smith and Webbon 1996; Riley and Bailey unpublished data). The relative proportions of these cell types and their overall density in the ECM varies according to the tendon of origin, the segmental location of the cells (ie. zones subjected to predominantly tensile forces compared to zones subjected to primarily compressive forces), and the age, sex and type of the horse from which the tendon is obtained (Holmes 1971; Otomo 1973; Webbon 1978; Gelberman *et al* 1984; Smith and Webbon 1996; Bailey *et al* 1996; Bailey *et al* unpublished data). Within the matrix of the SDFT, type I tenocytes are small (approximately 18 x 6 μm), have dark ovoid nuclei and scant amounts of pale staining cytoplasm (haematoxylin and eosin), and are responsible for synthesis, organization and maintenance of adjacent ECM components (Holmes 1971; Williams *et al* 1980; Doane and Birk 1991). In the endotendon regions there is a mixture of fusiform fibroblasts (including type II tenocytes) with plump nuclei and endothelial cells. The tendons of younger horses have greater numbers of type II cells between the collagen fibres and greater cell density generally, whereas in older horses the type I cells are more common and cell density lower, especially in the core regions (Webbon 1978; Goodship and Birch 1996; Riley and Bailey

unpublished data). In regions subjected to compression such as the metacarpophalangeal zone, there are more type III cells. In the tendons of older horses, or horses that have previously sustained an injury, aggregations of type III cells surrounded by cartilage-like matrix and/or calcified matrix may be observed (Webbon 1978). The cellular density of the equine SDFT has generally been shown to be greater than that of the DDFT, and the morphological characteristics of the tenocytes suggest that they are more metabolically active (Bailey *et al* 1992).

In studies using the chicken as a model of synovial sheathed tendon healing, techniques for the separation of tendon cell subpopulations and for the identification of those cells which are producing collagen in healing flexor tendons have been described (Riederer-Henderson *et al* 1983; Banes *et al* 1988; Garner *et al* 1989). Morphological, adherence and growth curve characteristics were determined which indicated that the surface and internal cells of avian flexor tendons are comprised of at least two different cell populations (Banes *et al* 1988). A surface population (synovial cells) derived from the chick epitendon was differentiated from internal tendon fibroblasts by location in the tendon, decreased susceptibility to trypsin, slower growth rate, and distinct morphology. The surface cell population consisted of large, round cells with ruffled plasma membranes and two classes of cytoplasmic inclusions. Conversely the internal fibroblast population appeared to be a more homogenous group based on morphological characteristics (Banes *et al* 1988). In one study the cells of the outer surface population (synovial cells) of sheathed tendons produced collagen types I and III, whereas the inner cells produced only type I (Riederer-Henderson *et al* 1983). Skin fibroblasts are capable of synthesizing both collagen types I and III (Gay *et al* 1976). The tenocytes have been shown to differ from other fibroblast-like cell types in their sensitivity to reduced oxygen tension as well as their *in vitro* responses to tissue culture (Webster and Burry 1982; Riederer-Henderson 1983).

The separation and investigation of cellular subpopulations within the equine SDFT has been hampered by the resistance of the tissue to chemical or enzymatic breakdown, the risk of damaging cells when mechanical techniques are used, and the low cellular density of mature tendon tissue. Collagenase digestion has been utilized to release intact tenocytes, but

this technique released only 0.3% of the total number of cells, mostly from the cut surface of the tendon (Birch 1993). Birch (1993) isolated tenocytes from the peripheral and central zones to evaluate and compare their anaerobic and aerobic metabolic activity. Cells from the central and peripheral zones were found to have similar morphology and enzyme profiles. With the exception of pathologic studies, there have been few other published reports investigating the morphological or functional characteristics of subpopulations of tenocytes in the equine SDFT (Webbon 1978; Goodship and Birch 1996).

It has long been known that cell density is a key determinant of the rate of cell proliferation and collagen synthesis by fibroblasts *in vitro* (Freshney 1987; Schwarz 1991). However, investigation into intercellular signalling mechanisms between tenocytes is in its infancy. Cells from primary cultures of avian tendon are able to detect changes in cell density over ~1 mm distance via a 'cell density signal', and intercellular signalling and therefore procollagen synthesis, can be disrupted by mechanically agitating the cultures (Schwartz 1991). More recently gap junctions were demonstrated in rat tail tendon and canine patellar and digital tendons in situ by transmission electron microscopy and immunoflourescent labeling (Ciarelli *et al* 1996). It is believed that these junctions may be part of an intercellular communication network enabling the tenocytes to respond to their mechanical environment. The author is unaware of any publications which have investigated intercellular signalling in the equine tendons or their constituent cells.

2.4.2 Energy Metabolism of Tenoctyes

Basic knowledge pertaining to the ultrastructural composition and enzyme activities of normal tenocytes may be useful in the understanding of the pathogenesis of tendon disease. Currently there is a limited understanding of such processes in the tenocyte during development and ageing, or in response to injury or exercise (Landi 1987). As previously stated, it is the tenocytes which are responsible for maintaining the tendon through the synthesis (and possibly catabolism), aggregation and organization of the ECM (Doane and Birk 1991). Since both synthesis and transport of the ECM proteins are energy dependent

their investigation is of value for understanding the development and capacity for repair of the tendon (Birch 1993). In the equine SDFT is has been theorized that type II and type III cells are the more metabolically active of the tendon cells, although morphologic changes in the type I cells occur when explants are maintained *in vitro* for up to 30 days (Smith and Webbon 1996; Riley *et al* unpublished data). The majority of what is known of tenocyte metabolism has been obtained in the rabbit, while the equine SDFT has only recently been investigated (Landi *et al* 1980 a,b,c; Floridi et al 1981; Landi 1987; Birch 1993).

Birch *et al* (1993) investigated lactate dehydrogenase (LDH), citrate synthetase (CtS), maleate dehydrogenase (MDH) and glutamate dehydrogenase (GDH) activities of freshly isolated cells of the equine SDFT *in vitro*. LDH was used to assess anaerobic metabolism of glucose (glycolysis), and the latter three enzymes to identify the presence of mitochondria and the cellular potential for mitochondrial respiration (the citrate or Krebs cycle). Finally glucose utilization through glycolysis and the Krebs's cycle was assessed directly and it was found that tenocytes had an oxidative capacity comparable to that of other mammalian cell types (based on CtS, MDH and GDH levels of activity), were able to utilize glucose, and also had sufficient LDH activity to withstand short periods of low oxygen tension using anaerobic glycolysis (Birch 1993). However, in contrast to a previous study by Webster and Bury (1982), the latter finding did not suggest a greater anaerobic capacity for these cells than other cell types. In subsequent studies, both LDH and MDH activities were found to increase with longer *in vitro* culture periods, and the oxidative metabolism of glucose declined (Birch 1993). Furthermore, the cells depend upon anaerobic metabolism for the maintenance of intracellular ATP (energy) levels at least to some degree.

2.5 Cell Surface Receptors for the Extracellular Matrix

Contrary to earlier belief, the tendon is a dynamic structure capable of metabolic activity including cell proliferation, differentiation, migration and regulation of the synthesis and catabolism of the constituent molecules of the ECM (Fackelman 1973). Both the cells and the ECM may interact with and influence each other. Changes in the composition and

structure of the ECM must be continuously transmitted to and interpreted by the target cells and tissues (Raghow 1994; Goodship and Birch 1996). Conversely, cell orientation (ie. the linear arrangement of tenocytes parallel to the collagen fibres and lines of tensile force) and the mechanical forces to which they are subjected influence the synthesis, assembly and orientation of ECM (Norberg *et al* 1967; Stromberg 1971; Goodship *et al* 1980). Evidence of the activation of ion channels in response to mechanical stimuli such as stretch and compression which alter cell shape has been found (Guharay and Sachs 1984; Giori *et al* 1993). However, the initiation and regulation of these and other processes are poorly understood and there in little information on transmembrane receptors and the transmission of signals from the extracellular matrix and other tenocytes to the cell cytoplasm (Labat-Robert *et al* 1990; Ciarelli *et al* 1996).

2.5.1 The Integrins

The cellular response to the ECM is mediated by two major classes of surface receptors: (1) a family of over 20 heterodimeric cell adhesion receptors called the integrins, and (2) a family of unique cell surface associated PGs (Goetink 1991; Jackson *et al* 1991; Wight *et al* 1992; Hynes 1992; Juliano and Haskill 1993). The integrins are the principal transmembrane receptor proteins and are composed of two glycoprotein subunits which mediate cell surface interactions with other cells (in some instances) and link the ECM to the cells' cortical cytoskeletons. They are expressed by almost all normal cells but most cells express more than one type of integrin on their surface, with the unique cell and ligand specificity of each of these receptor proteins generated by greater than 20 various heterodimeric combinations of the 14 known α and nine β chain subunits (Raghow 1994; Yamada *et al* 1996). Additional variations in the specificity of the integrins are created from extensive alternative splicing of the precursor mRNA molecules of the constituent α and β chains (Raghow 1994; Yamada *et al* 1996). Some integrins bind only one ECM molecule such as fibronectin or laminin. Others, including a fibroblast integrin which binds fibronectin, laminin and collagen, recognize more than one ECM molecule type (Raghow 1994; Alberts

et al 1989). Some ECM proteins may be recognized by multiple integrins, and the sequences or domains that are recognized within these molecules may vary for different integrins, or for different cell types.

The integrins differ from other cell surface receptors such as those that bind hormones, growth factors and other small signalling molecules because they bind with relatively low affinity and are present in concentrations that are 10-100 fold greater on the cell surface. This arrangement permits the cell to bind weakly to large numbers of matrix molecules, so that they may move readily without losing contact with the ECM. Differences in the integrins and their ligand-binding specificity are expressed by different cell types and reflect their ability to migrate within and interact with the ECM (Yamada *et al* 1996).

The β_1 chains form heterodimers with at least nine different α chains and are found on most vertebrate cells. Examples include the $\alpha_1 \beta_1$ and $\alpha_2 \beta_1$ integrins which bind native collagen, $\alpha_5 \beta_1$ which is a fibronectin receptor and $\alpha_6 \beta_1$ which is a laminin receptor, all of which are found on most connective tissue cell types (Faull *et al* 1993; Gailit and Clark 1994; Yamada *et al* 1996). The β_2 integrins are responsible for mediating cell-cell interactions and are expressed only by white blood cells. The β_3 integrins are found on a variety of cells including platelets, permitting their interaction with fibrinogen, and endothelial cells ($\alpha_v \beta_3$ integrin), facilitating angiogenesis (Gailit and Clark 1994; Alberts *et al* 1989).

The integrins are the principal transmembrane receptor proteins which mediate interactions that link the ECM to the cell cytoskeleton. This is facilitated by the extracellular and intracellular domains of the integrin molecules. Activation of an integrin may result in reorientation of the extracellular receptor domain, following which the linking of this domain to its ECM ligand results in binding of the cytoplasmic tail of the beta chain to talin and α-actinin. This initiates the formation of a complex of intracellular assembly proteins that link the integrin to the actin filaments of the cytoskeleton, resulting in focal contact between the ECM and the cortex of the cell (Juliano and Haskill 1993). Thus the ECM can influence the organization of the cell's cytoplasm (eg. the tenocytes are aligned in the same direction as the collagen fibres), and conversely the intracellular actin fibrils can influence the orientation of secreted fibronectin molecules (Alberts *et al* 1989). The adhesive activity of each cellular

integrin is regulated by changing either the conformation of the extracellular domain or their intracellular attachment to actin filaments. The exact mechanism of these regulatory processes are unknown (Alberts *et al* 1989). Nevertheless, the interrelationship of the cytoskeleton and ECM molecules permits the propagation of forces from cell to cell, and the orientation of larger structures (eg.tendons), with the integrins mediating these processes. In addition to their structural role as transmembrane linkers, the integrins are also capable of activating intracellular signalling cascades, thereby permitting ECM molecules to influence the behaviour of cells in culture, their shape , their polarity, movement, metabolism, development and differentiated functions (Alberts *et al* 1989).

2.5.2 The Cell Surface Associated Proteoglycans

Proteoglycans are glycoproteins to which one or more polysaccharide chains are attached. Although the majority of the well characterized PGs occur in the ECM, there are some forms which remain and function intracellularly (eg.serglycin), and still others which are integral components the cell plasma membrane (Jackson *et al* 1991; Wight *et al* 1992). Of the latter category, the best characterized is syndecans which has a transmembrane protein core (~ *mw* 32,000) with intracellular and extracellular domains (Alberts *et al* 1989). The intracellular domain interacts with the actin cytoskeleton and the extracellular domain has a variable number (one to three) of heparin sulfate (HS) and CS GAG sidechains, giving it an anionic charge density suitable for binding a variety of charged molecules (Wight *et al* 1992). Syndecans is found in fibroblasts and many other cell types, and has a similar role to the integrins in binding collagen, fibronectin and other matrix proteins (Alberts *et al* 1989). Syndecans also acts as a co-receptor by binding fibroblast growth factor (FGF) to its HS sidechains, and then presenting it to the appropriate FGF receptor proteins on the same cell. Other transmembrane PGs such as betaglycan (which also occurs on a soluble form in the matrix that is not associated with the plasma membrane) bind transforming growth factor beta (TGF-ß) and presents it to the TGF-ß receptors (Alberts *et al* 1989). However in the case of betaglycan, TGF-ß binds directly to the core protein, unlike the non-specific binding

to the GAG chains seen with other growth factors (Boyd *et al* 1990). Hence betaglycan is believed to regulate the clearance or storage of TGF-ß, releasing it when the matrix is damaged.

2.6 The Extracellular Matrix of Connective Tissues

Once thought to be an inert scaffolding functioning only to stabilize the physical structure of tissues, the ECM of the connective tissues is believed to play a crucial role in tissue development, function and repair (Alberts *et al* 1989). The tendons have a very high ratio of ECM to cells, and the relative scarcity of cells within the adult equine SDFT compared to its muscular origin, implies that turnover of this component of the normal tendon occurs at a slow rate (Chvapil 1996; Bailey *et al* unpublished data). Tendon tissue is structurally diverse in its composition, with variations in matrix components corresponding to the various tensile and compressive forces to which it is likely to be subjected (Okuda *et al.* 1987; Jones and Bee 1990; Abrahamsson 1991; Bailey *et al* 1992; Riley *et al* 1995; Bailey *et al* 1996; Crevier *et al* 1996). All connective tissue matrix consists of at least four major types of macromolecules: collagen; elastin; PGs (and nonsulphated GAGs); and connective tissue glycoproteins (Birch 1993). The tendon is a stress-resisting system in which the fibrillar collagen component resists pulling (tensile) forces and the inter fibrillar PGs and HA gel resist compressive forces (Scott 1990). The fibrous proteins of the ECM constitute two major subclasses: the structural proteins, of which collagens and elastin are the major molecules present, and the adhesive proteins like fibronectin and laminin that are associated with the migration and attachment of cells, and are important in wound healing (Abrahamsson 1991; Yamada and Clark 1996). The PGs contribute to water content and the resiliency of the tissue, and are thought to be important in regulation of collagen fibril formation and other ECM and cellular interactions (Parry *et al* 1982; Vogel *et al.* 1984; Scott 1990). In the equine SDFT, the water content has been determined to be 65-68% by weight, compared to 55% for the rabbit, 54-57% for the dog and 70% in the human digital tendon (Okuda *et al* 1987; Abrahamsson 1991; Birch 1993; Bailey *et al* 1996).

2.6.1 Collagen Structure and Synthesis

Collagen constitutes a major portion of total body protein, with the concentration in the normal adult equine SDFT reported at 64-80% of the dry weight, with marked horse to horse variation (Williams *et al* 1980; Birch 1993; Riley *et al* 1995; Bailey *et al* 1996). It has been suggested that the tendon is a dynamic structures in which the cells may renew all its collagen every six months (Fackelman 1973). There are no published reports of research substantiating this claim in the horse but it is clear that high rates of collagen synthesis and catabolism do occur during healing after tendon injury (Chvapil 1996; Clark 1996).

Molecular Structure

Collagen molecules are the basic unit for the tendon collagen fibre and the essential moiety for the resistance of tensile force. Each molecule consists of three polypeptide α-chains, wrapped around each other in a right handed superhelix with a pitch of about 39 residues (89 Å -units) (Kuhn 1987; Nimni and Harkness 1988). Each subunit polypeptide α-chain is formed into a left handed helix with 3.27 amino acid residues per ~110° turn, resulting in a 2.91 Å displacement between consecutive amino acids, and a 8.7 Å distance between each third glycine (Nimni and Harkness 1988; Parry 1988). This separation prevents intrachain bonds from forming, leaving sites open for interchain hydrogen bonds (one to two bonds per amino acid triplet) to form and stabilize the triple chain supercoil (Nimni and Harkness 1988). There are at least 20 different α-chains which have been identified as subunits of at least 15 different collagen molecules, although new members of the collagen family are still being identified and used as markers for different connective tissues (table 2.1) (Kühn 1987; Jones and Bee 1990; Abrahamsson 1991).

The three α chains which form the triple helix of the rod-like collagen molecule each contain a substantial proportion of amino acid sequences in a repeating triplet of glycine (Gly) and two other amino acids (Gly-X-Y), where X and Y may be any amino acid, but are most frequently proline (Pro) in the X position, and hydroxyproline (Hyp) in the Y position

(Parry 1988; Bailey and Light 1989; van der Rest and Garrone 1991). For type I collagen, two of the three α chains are identical (α_1(I)) and the third α_2 is homologous but chemically distinct (table 2.1) (Kuhn 1987). The triplet region of both chains extends for a length of approximately 1000 residues, terminated at either end by short sequences called telopeptides (Parry 1988). The imino acids Pro and Hyp are particularly important for the stability of the helix; the order of stability for imino acid containing tripeptides being: Gly-Pro-Hyp (most rigid) > Gly-Pro-Y and > Gly-X-Hyp (least rigid) (Kuhn 1987). Therefore the rigidity and flexibility of the molecule can be modified by varying amounts of Pro and Hyp incorporated into the collagen molecule.

Table 2.1: Characteristics of major and minor collagens in tendon tissue

Type	Chains	Molecular structure	Supermolecular tertiary structure	Location
I†	2[α_1(I)],[α_2(I)]	300 nm	67 nm banded fibres	matrix
III†	3[α_1(III)]	300 nm	small 67 nm banded fibrils	matrix & endotendon
IV†	α_1(IV), α_2(IV)	390 nm C globular domain	nonfibrillar sheet-like tetramer network	basement membranes
V†	α_1(V),α_2(V), α_3(V)	300 nm N globular domain	small fibrils	basement membranes
XII‡	3[α_1(XII)]	globular with 75 nm tail & 3x60 nm arms	nonfibrillar complex; fibril associated	unknown
XIV‡	3[α_1(XIV)]		nonfibrillar complex, fibril associated	unknown
fp55‡	unknown	unknown	unknown	unknown

† = major , ‡ = minor constituent; (Kuhn 1987; Amiel and Kleiner 1988; Dublet *et al* 1989; Dublet and van der Rest 1991; Jones and Bee 1990; van der Rest and Garrone 1991)

Hydroxyproline is important in the formation of the interchain hydrogen bonds which stabilize the collagen molecule. Hydroxylysine is unique to collagen and is associated with intermolecular covalent cross-links within the telopeptides (with the exception of the C-terminal end of the α_2 chain) (Parry 1988). A deficiency in Hyp results in reduced tensile strength of the tendon (Abrahamsson 1991). The intermolecular hydrogen bonds stabilizing the triple helix extend from the NH of a glycine residue to the back-bone C=O of the X residue in the adjacent chain, stabilizing each molecule with approximately 1000 such bonds per collagen molecule (Birch 1993). The hydroxylation of Pro and lysine occurs after incorporation into the respective amino acid into the peptide chain, and is catalysed by enzymes that require free oxygen, ferrous iron, alpha ketoglutarate and ascorbate (Kuhn 1987; Abrahamsson 1991). The enzymes that catalyse these reactions, proline 4-hydroxylase and lysyl hydroxylase, facilitate the oxidation of residues in position Y of the tripeptide unit Gly-X-Y (Kuhn 1987). There is also a prolyl-3-hydroxylase which oxidizes Pro residues in the X position (Abrahamsson 1991). Differences occur between different collagen types in the concentrations of Hyp, Pro and other amino acids present in the X and Y positions, an thus, the overall molecular and tertiary aggregate structure of their helices (table 2.2). Proline ranges from approximately 13% of all amino acids by weight for type I collagen to 17.4% for type III collagen (Bailey and Light 1989). In the rabbit and the cow, Hyp has been estimated to constitute 78-118 mg/g of tendon dry weight, with variability reported due to age and tendon location (Manske and Lesker 1984; Koob and Vogel 1987a). Although very small amounts are present in elastin and acetylcholine, Hyp is almost unique to collagen and is not amenable to metabolic recycling by the cell.

Table 2.2: Amino acid composition of the α-chains of the major collagens in tendon tissue[†].

Amino acid	Collagen Type						
	Type I		Type III	Type IV		Type V	
	$\alpha_1(1)$	$\alpha_2(1)$	$\alpha_1(III)$	$\alpha_1(IV)$	$\alpha_2(IV)$	$\alpha_1(V)$	$\alpha_2(V)$
Glycine	352[‡]	350	354	318	313	318	320
Proline	139	123	109	79	69	92	119
Hydroxyproline	102	87	127	146	127	107	108
Alanine	121	113	97	31	313	318	320
Glutamine	74	69	72	77	62	90	99
Arginine	51	53	47	20	45	57	48
Asparagine	43	46	43	48	52	55	51
Serine	39	36	39	34	26	34	26
Lysine	31	23	30	5	6	18	19
Valine	21	34	14	30	24	30	19
Leucine	21	33	22	54	59	37	40
Threonine	17	19	13	18	28	27	22
Phenylalanine	13	12	8	24	37	12	12
Isoleucine	7	15	13	34	42	18	20
Methionine	7	5	8	15	12	10	7
Hydroxylysine	5	9	5	61	42	25	36
Histidine	2	11	6	6	8	10	7
Tyrosine	2	4	3	5	6	-	1
Cysteine	-	-	2	-	-	-	-

[†]based on values reported by Bailey and Light (1989); [‡]numbers represent approximate number of residues per α–chain molecule.

Collagen Types

The predominant structural collagen types I, II, III and V are present in many tissues, but it is the variation in occurrence and distribution of the minor collagen types which assist in distinguishing connective tissues previously considered as identical (Gordon *et al* 1987; Amiel and Kleiner 1988; Jones and Bee 1990; Goodship *et al* 1994). Types I, II, III, V and XI form tertiary aggregates of quarter staggered fibrils, whereas types XII, XIV and the GAG-linked type IX (which assembles around type II fibrils in hyaline cartilage) are fibril associated with interrupted triple helices (the FACIT collagens) (van der Rest and Garrone 1991; Raghow 1994). Collagen types IV, VIII and X form into sheets of lattice-like sheets of tetramers ('spiders'), type VI forms beaded filaments of serially linked tetramers, and type VIII aggregates into dimers which act as anchoring fibrils. Collagen type I is the primary fibrous structural protein of adult equine tendon (~95% of all collagen), irrespective of intrinsic morphological or age variation (Williams *et al* 1980; Silver *et al* 1983; Parry 1988; Jones and Bee 1990; Goodship *et al* 1994; Smith and Webbon 1996).

The second most predominant collagen in tendon, type III (~ 5%), surrounds type I fibres regardless of fibril diameter, and is linked covalently in tendon to type I collagen by lysine derived cross-links (Keene *et al* 1987). It is also found in the endotendon regions and in tendon sheaths and has smaller fibril diameters than type I (Duance *et al* 1977; Birch 1993; Smith and Webbon 1996). Increased type III collagen production occurs in association with tendon healing, and has been identified in conjunction with increased cellularity in equine tendon scars of the intratendinous regions of the SDFT (Silver *et al* 1983; Watkins *et al* 1985a; Watkins *et al* 1985b). It also occurs in high concentrations in the immature tendons of the fetal and neonatal horse (Williams *et al* 1980). Collagen types IV and V are pericellular collagens that have been detected in association with basement membranes and the fine reticulate network of endotendinous sheaths and embryonic tendons of the chick (Duance *et al* 1977). Type IV usually occurs in all basement membranes together with the glycoproteins laminin, nidogen, entactin, and HS-PG, and tends to form aggregates in the form of sheets rather than the quarter staggered fibrils that occur with types I, II, III, V and

XI collagen (Glanville 1987; van der Rest and Garrone 1991). Collagen type V has been reported peripherally adjacent to the endothelial cells of capillaries and functions as a connector between basement membranes and stroma (Watkins *et al* 1985a; Fessler and Fessler 1987). It may also be closely associated with type I collagen during fibre formation (Fessler and Fessler 1987). The presence of both types III and V collagen molecules have been shown to have a direct effect on the fibril diameter of other fibrillar collagens (Adachi *et al* 1989). Collagen types IV and V have been identified in the pericellular regions of healing equine SDFT following the creation of a surgical lesion (Watkins *et al* 1985a; Watkins *et al* 1985b). Although these types were detected at weeks two, four, eight and twelve following injury, they were not detected at week 24, and their occurrence and distribution in normal equine tendon has not been reported. A novel collagen type XII has also been identified in chick embryo tendons (Sugrue *et al* 1987). Type XII collagen has not yet been identified in equine tendon, but recently two proteins have been isolated from fetal SDFT (np 52 with molecular weight 52 kD and np 54 with molecular weight 54 kD) and another from the fetlock region of the adult SDFT (fp 55 with molecular weight 55 kD) (Jones and Bee 1990). The fp 55 was found to be collagenase resistant and was thought to have homology with other collagen types but details of its precise nature have not been published. The equine SDFT may contain other unidentified unique minor collagens and further investigation may identify these (Jones and Bee 1990).

Collagen Synthesis and Molecular Aggregation

The large genes (of which 10-30% of each codes for the protein) responsible for synthesis of the fibrillar collagens have similar intron/exon structures and are well conserved across the species (Boedtker *et al* 1983). The fibrillar collagens are synthesized initially as pre-procollagen molecules with large amino- (N-) and carboxy- (C-) terminal globular domains which are not deposited in the final fibrillar structure (figure 2.1). The triple helical domain, including the non-triple-helical segments at either end, contains all the structural data for the construction of a cross-linked fibril in the extracellular space (Kühn 1987).

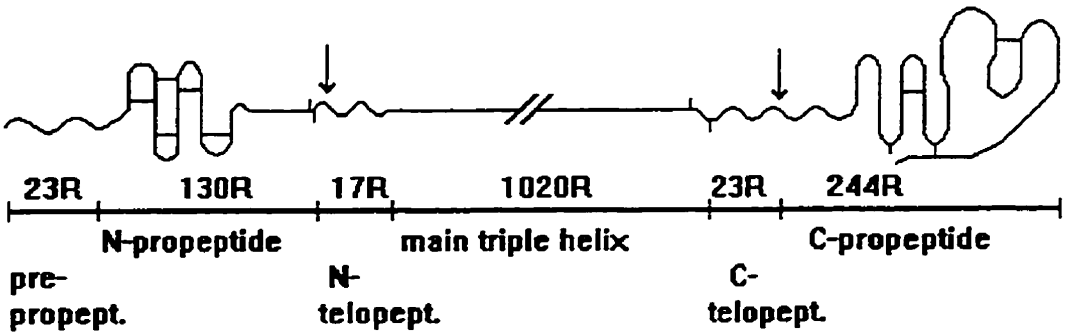

Figure 2.1. Representation of the prepro α_1(III) chain of collagen (Kuhn 1987). The cleavage sites of the amino and carboxyl procollagen peptidase are indicated by the arrows. R = amino acid residues.

Newly synthesized pre-procollagen polypeptide chains contain a short hydrophobic signal peptide of 23 amino acids located at the N-terminal which facilitates the penetration of the α-chains through the rough endoplasmic reticulum after which the peptide is cleaved off (Kühn 1987).

The hydroxylation of Pro and lysine residues is crucial to collagen biosynthesis and occurs in the endoplasmic reticulum. Hydroxylysine may then become glycosylated by the addition of galactose, catalysed by hydroxylysine galactosyl transferase, or by the addition of galactose and then glucose via the catalytic enzyme galactosyl hydroxylysine glucosyl transferase (Kivirikko and Myllyla 1979). The level of glycosylation is low for collagen types I and III with only 0.5-1% of hydroxylysine residues transformed. The carbohydrate residues affect the amount of associated water and therefore fibril spacing. The C-terminal propeptide has eight cysteine residues, a proportion of which are associated with interchain disulphide bonds catalysed by disulphide isomerase. This aligns the polypeptide chains in order and initiates triple helix (tropocollagen) formation (Fessler *et al* 1981).

The energy dependent secretion of newly formed procollagen by exocytosis is followed by the cleavage of the amino- and carboxy-terminal residues by specific peptidases just before or during fibrilogenesis. The presence of the N- and C- propeptides prevents intracellular fibril formation (Kühn 1987). They are important for the post translational

modification of collagen, and possibly for feedback regulation of collagen synthesis (Fessler *et al* 1981; Wiestner *et al* 1979).

The molecular packing of collagen molecules is an important determinant of the mechanical strength of the tendon. Once the propeptides are cleaved the collagen molecules have the capacity to self assemble into microfibrils under the influence of local tenoblasts (Birk and Zycband 1994). The plasma membrane of these cells is complex and compartmentalizes the extracellular space to create defined regions in which collagen fibril, bundle and macroaggregate formation can occur. All these phases of structural development of the collagen matrix occur in close association with the tenoblasts (Birk and Zycband 1994). The microfibrils are packed into a tetragonal lattice with a lattice period of 3.8 nm leading to the collagen fibril (Baer *et al* 1974). X-ray diffraction techniques and electron microscopic examination of collagen fibrils in tendon have determined that a periodicity exists of about 67 nm (*D*) (Parry 1988). However, the length of the collagen molecule including the telopeptides (~300 nm or 4.4 *D*) is not an integral multiple of the *D*-period, implying that individual molecular structure has alternating regions of dense molecular packing (overlap regions of 0.47 *D*) and less dense molecular packing (gap regions of 0.53 *D*). Statistical evaluation of the amino acid sequences of α_1(I), α_2(I), and α_1(III) polypeptides indicated that the 234-amino-acid-residue-long unit was the most prominent repeat and is equivalent to 67 nm (*D*) observed on electron micrographs (Kühn 1987; Parry 1988). It is formed predominantly by polar charged and hydrophobic residues and divides the α chains into four homologous parts: D1, D2, D3, and D4 (Hofmann *et al* 1978; Hulmes and Miller 1979). Three dimensional calculations of the interaction between collagen I and III molecules, indicates that the highest polar and highest hydrophobic contact occurs when the individual molecules are shifted against each other by a distance of 234 amino acid residues (Hofmann *et al* 1978). The interaction of neighbouring molecules allows the formation of ordered clusters of ions and hydrophobic side chains giving greater stability than possible with only single residue interactions (Parry 1988). This appears to be important for axial stability of the fibrils. The lateral array of the fibril is also important, but it is not as strongly determined by the amino acid sequence as is the axial stagger of the molecules.

Fibril Growth and Development

The morphology of collagen fibrils has been examined with the electron microscope through stages of development ranging from the early fetus through to maturity and this has been described in section 2.1. Initially collagen appears as widely dispersed bundles of limited size containing small constant diameter fibrils. Over time, the lateral dimensions of the bundles increase without an increase in the diameter of its constituent fibrils. Hence the number of fibrils increases and it is assumed, but not confirmed, that fibril length increases (Kühn 1987). During the next stage of development, fibrils in neighbouring bundles often have different mean diameters. The fibril diameter distribution widens and each bundle is composed of populations of fibrils of discrete sizes. This occurs prenatally in precocial animals and postnatally in altricial animals. Finally, collagen fibril diameter distribution becomes unimodal or bimodal in tendon as the animal matures (Parry 1988). There is little data on fibril length due to the difficulty associated with preparing, mounting and examining entire fibrils. However, based on transmission electron micrographic studies, a mechanism for lengthening and lateral enlargement of fibrils has been proposed (Birk and Zycband 1996). Microfibrils first enter the extracellular space in deep narrow channels or invaginations in the plasma membrane of the tenoblasts (close to the Golgi region) which open to the extracellular space. As the microfibrils move out of these invaginations and enter the extracellular space proper, it has been proposed that new microfibrils enter the vacated space and are fused onto the microfibril produced earlier, enabling lengthening in the case of linear fusion, and increase in diameter of a larger fibril in the case of lateral fusion (Birk and Zycband 1996). Mean fibril lengths of 125μm and 1700μm have been reported for 5-day-old and 4-month-old rats respectively, but are not reported for the horse (Parry 1988).

Mature equine SDFT has been reported to have a bimodal distribution of fibril diameters with the mean diameters of the two peaks equal to 35 and 215 nm (Parry 1978). Following injury to the SDFT small fibrils predominate for 14 months or more (Silver *et al* 1983). Recent work in the horse has established and compared some of the fibril characteristics of the equine SDFT and DDFT, and examined the effects of exercise on fibril

diameter (Bailey *et al* 1992; Birch 1993; Patterson-Kane *et al* 1997b). A comparison of mean fibril diameter and fibril distribution found no significant differences between peripheral and central zones of the SDFT. However, the mean diameter of the fibrils in the SDFT (median 48 nm, range 9-364 nm) was significantly lower than that of the DDFT (median 123 nm, range 18-458 nm) (Bailey *et al* 1992). Ninety percent of fibrils in the DDFT had diameters greater than 150 nm whereas only 55% of those in the SDFT were greater than 150 nm in diameter. It has been suggested that these differences may reflect the different functions performed by the two tendons and differences in their level of developmental maturity (Birch 1993). When a group of 18-month-old Thoroughbred horses given a controlled exercise program for 18 months, fibril diameters were decreased compared to unexercised controls in the central mid-metacarpal regions of the SDFT (Patterson-Kane *et al* 1997b).

Collagen Cross-links

There are specific regions along the triple helix which are important for the stabilization of the collagen fibril by cross-linking, for resistance of fibril degradation by collagenase, and for the interaction of collagen with cells and other matrix molecules (Kuhn 1987). The intramolecular and intermolecular cross-links contribute greatly to the high mechanical strength of collagen fibres (and elastin fibres), and the tendon as a whole (Eyre *et al* 1984). The three major types of cross-links are disulphide bridges, lysyl oxidase mediated cross-links and those resulting from the non-enzymatic glycosylation of lysine and hydroxylysine residues (Bailey and Light 1989).

Disulphide bridges occur only in collagen molecules with cysteine residues (eg. type III collagen). In type III collagen the cysteine residue in the final triplet before the C-terminal domain of the triple helix is the site of cross-link formation (Birch 1993). These bridges do not contribute significantly to the mechanical strength of the collagen fibres.

Lysyl oxidase mediated cross-links are the major covalent cross-links in type I collagen and essential determinants of the tensile strength, and resistance to chemical or enzymatic breakdown of collagen (Reiser and Last 1986; Amiel and Kleiner 1988; Smith and

Webbon 1996). These enzyme mediated reactions involve mainly lysine and hydroxylysine which have secondary amine groups on terminal projections extending lateral from the α-chains and are available for cross-linking (Amiel and Kleiner 1988). Inhibitors of lysyl oxidase such as ß-cyanoalanine and its decarboxylated product ß-aminoproprionitrile, and D-penicillamine result in decreased cross-linking and lathyrism (Amiel and Kleiner 1988; Chvapil 1996; Davis 1996). Cross-linking sites are characterized by low Pro and Hyp content and the presence of the sequence Hyl-Gly-His-Arg, which is an important attachment site for the enzyme lysyl oxidase (Kuhn 1987).

A third group of nonenzymatically derived glycosylated lysine and hydroxylysine residues occurs in collagen (Eyre *et al* 1984). These residues are thought to be sugar derived cross-links, but the biochemical pathways are poorly understood, and their importance as cross-links in tendon is not known (Birch 1993). Others have suggested that these residues are not cross-links, but molecules which complicate the 'cross-linking profile' which are elevated in concentration with age and diabetes mellitus (Eyre *et al* 1984) .

2.6.2 Elastin

Elastin is a inert, amorphous protein of approximately 750 amino acid residues capable of high degrees of reversible extension. It is a highly hydrophobic protein which has a high concentration of Gly and Pro residues (like collagen), but unlike collagen, it has little Hyp (<1.5%) or hydroxylysine, and is not glycosylated (Bailey and Light 1989). Although it is a major constituent of blood vessels and some ligaments (from 5% in most ligaments to 70% of the nuchal ligament of ruminants), it occurs in low concentrations in the equine SDFT, predominantly in the blood vessel walls of the endotendon (Bailey and Light 1989; Smith and Webbon 1996). Following exocytosis, the elastin molecules conglomerate into fibres which then become cross-linked to one another to form fibres and sheet-like networks. Turnover rate is very low and the molecules are extremely resistant to degradation due, in part, to extensive cross-linking which occurs during its maturation (Eyre *et al* 1984). Unlike collagen, the elastic fibre is formed from two components. In the mature elastic fibre the

major component is elastin, but during development, a 10 to 12 nm diameter microfibrillar protein is detected at the periphery of the amorphous elastin and becomes progressively enveloped in elastin (Bailey and Light 1989). The enveloped microfibril appears to function as a template during elastic fibre development only, constitutes only a small percentage of the mature fibre, and does not contribute to the mechanical qualities of the mature fibre.

2.6.3 Proteoglycans and the Glycosaminoglycans of the Extracellular Matrix

Proteoglycans Structure and Classification

The proteoglycans constitute a group of complex macromolecules containing one or more sulfated GAG side chains covalently linked to a core protein either through: (1) *O*-glycosidic linkage between D-xylose and the hydroxyl group of serine (unique to proteoglycan) , (2) an *N*-glycosamine linkage between *N*-acetylglucosamine and the amide group of asparagine residues, or (3) an *O*-glycosidic linkage between *N*-acetylgalactosamine and the hydroxyl groups of serine or threonine (Rodén 1980; Scott 1988; Raghow 1994). It is the presence of the one or more polyanionic GAG side chains (due to sulfate and carboxylate residues) which serves to differentiate the PGs from other glycoproteins (Rodén 1980). The PGs may occur intracellularly in secretory vesicles (eg. the serglycin PGs), at cell surfaces where they cross the plasma membrane an act as cell receptors and co-receptors (see section 2.4.2), and in soluble form as part of the structure and signalling apparatus of the ECM (Jackson *et al* 1991; Wight *et al* 1992; Gallo and Bernfield 1996). The major function of PGs is to carry GAG chains which impart strongly hydrophilic properties on the PGs, increasing the ability of matrix to bind water, particularly where tissue is required to resist compression (Vogel and Heinegard 1985). Therefore the regulation of transport and expression of the protein core of the PGs in turn determines the availability and type of GAG chains (Gallo and Bernfield 1996). In addition to this function, the PGs are recognized as regulators of cell behaviour via their interactions with growth factors (eg. the core proteins of biglycan and decorin bind directly to TGF-ß and therefore regulate its activity and

storage), and regulators of the synthesis of PGs and collagen, and the macromolecular assembly of fibrils (Handley and Lowther 1977; Boyd *et al* 1990; Gallo and Bernfield 1996; Gu and Wada 1996). In addition, there are specific peptide sequences within the core protein which bind other proteins such as link protein, or the metalloproteinases which are capable of degrading complexes of PGs. There is marked heterogeneity in the proteoglycan family of molecules, because structure may vary depending upon the differential expression of genes encoding their core protein, and the number, type and length of the GAG chains attached during the post translational modifications of the core proteins (Goetinck 1991). The protein cores vary markedly in size (ranging in molecular weight from 10,000 to 600,000), and it has been suggested that the PGs be categorized on the basis of molecular weight (small, large or very large) or on the basis of their predominant GAG side chains (Scott 1988; Gallo and Bernfield 1996; Smith and Webbon 1996). To date, very few of the core proteins of PGs have been isolated and their structure characterized (Jackson *et al* 1991).The small leucine-rich PGs such as biglycan, decorin and fibromodulin are globular proteins with one or two GAG sidechains. The large PGs have one or more globular regions and a linear polypeptide extension to which five to ten sulfated GAG chains are attached. Very large PGs (of which aggrecan the major PGs of cartilage, is the most widely studied) have up to three globular regions attached to a long polypeptide chain to which as many as 100 glycan chains are attached and can form large aggregates with hyaluronic acid (table 2.3) (Scott 1988).

Proteoglycans Synthesis and Catabolism

The synthesis of the proteoglycan core protein on membrane-bound ribosomes is followed by threading into the lumen of the rough endoplasmic reticulum (McQuillan *et al* 1986). The biosynthesis and addition of the many complex carbohydrate chains and sulfated GAG side chains onto the core proteins which possess the specific amino acid sequence, Serine-Glycine dipeptide, occurs mostly in the Golgi apparatus (McQuillan *et al* 1986; Yanagishita 1993). This is initiated by a special link tetrasaccharide which first attaches to a serine residue on the core, acting as a primer for the serial addition of sugar residues by

hexose-specific glycosyl transferases and sulfotransfeases. Sulfation of the GAGs occurs after the sugar residue has been incorporated into the growing chain. The sulfotransferases transfer sulfate groups from 3'-phosphoadenosyl-5'phosphosulfate to the GAG chain at specific sites (Champe and Harvey 1994). Following extrusion into the extracellular space the binding of the PGs (up to 100) molecules to hyaluronate and link protein results in the formation of aggregates which, with the water they bind, form the bulk of the non-collagen portion of the ECM.

Table 2.3: The major types of proteoglycans identified in the extracellular matrix of connective tissues.

Proteoglycan	**Major GAG constituents**	**Major cellular distribution**	**Reported functions**
Aggrecan [HA]	CS, KS; many chains	Chondrocytes	binds water in cartilage, resists deformation
Versican [HA]	CS; 12-15 chains	Fibroblasts	binds water in fibrous tissue
Decorin [L]	DS; 1 chain	Fibroblasts	fibrilogenesis control, binds TGF-ß, fibronectin
Biglycan [L]	DS; 2 chains	Fibroblasts	binds TGF-ß
Fibromodulin [L]	KS		binds TGF-ß
Lumican [L]	KS		binds TGF-ß
Perlecan [BM]	HS; 1-3 chains	Fibroblasts,endothelia, epithelia	organizes basement membrane, binds bFGF

[HA]hyaluronate and link protein associated; [BM]basement membrane associated; [L]leucine rich. (Jackson *et al* 1991; Kresse *et al* 1993; Gallo and Bernfield 1996).

The degradation of PGs occurs both intracellularly and extracellularly. Within the extracellular space the protein core is subject to a number of proteinases which act at specific sites resulting in its degradation. These metalloproteinases will be discussed in a following section. The degradation of the GAGs, on the other hand, occurs intracellularly following phagocytosis. The phagosome fuses with a lysosome and a number of hydrolytic enzymes cleaves the polysaccharides first into oligosaccharides, then sequentially by specific uronidases, sulfatases and transferases into simple hexoses (Gallo and Berfield 1996).

Sulfated Glycosaminoglycans

The sulfated GAGs are polysaccharide chains (usually unbranched) of varying length that are composed of repeating disaccharide units, one of which is always a hexosamine (N-acetylglucosamine or N-acetylgalactosamine) and the other either a uronic acid (glucuronic acid or iduronic acid) or galactose (Abrahamsson 1991). The acetylation of the amino sugars eliminates their positive charge, and the carboxyl groups of the uronic acids and the sulfate groups of the hexosamines impart a highly negative charge to the chains (Champe and Harvey 1994). The sulfated GAGs tend to be extended in solution, binding water and repelling each other so that when compressed, water is expelled, giving the tissue resilience. The family of sulfated GAGs include dermatan sulfate (D-glucaronic or L-iduronic acid + *N*-acetylgalactosamine-4-sulphate = DS), chondroitin sulfate (D-glucuronic + *N*-acetylgalactosamine sulfated either in the four or six position = CS), keratin sulfate (D-galactose + *N*-acetylglucosamine-6-sulphate = KS), heparin sulfate or heparin (*N*-acetylglucosamine-6-sulphate + L-iduronic acid or D-glucaronic acid = HS or H respectively), and hyaluronic acid (D-glucuronic acid + *N*-acetylglucosamine = HA). The GAGs possess the ability to bind covalently to a wide variety of molecules including protease inhibitors (eg. antithrombin III), plasma lipoproteins, growth factors (eg. FGF), adhesive and structural proteins of the ECM (Jackson *et al* 1991). Chondroitin sulfate and DS are galactosaminoglycans. These GAGs have considerable variation in the extent of sulfation resulting in significant heterogeneity (Gallo and Berfield 1996). Produced by many cell types,

CS is the most abundant GAG in mammals, and is the predominant GAG of tendon, cartilage, ligaments and the aorta (Birch 1993; Champe and Harvey 1994; Bailey *et al* unpublished data). It commonly occurs in large aggregates of CS-PGs (aggrecan in cartilage, and versican in tendon or ligaments) which are linked to hyaluronate and stabilized by their associated link proteins. In the ECM the role of the CS rich PGs is considered to be largely structural, giving the tissue resilience to mechanical forces, particularly those which are compressive in nature. It is also an important constituent of the intracellular and transmembrane PGs (Gallo and Berfield 1996).

Dermatan sulfate has many similarities to CS and is found in skin, blood vessels and heart valves, as well as tendons (Champe and Harvey 1994; Bailey *et al* unpublished data). It is not an important constituent of the intracellular and transmembrane PGs nor the large PG aggregates (Gallo and Berfield 1996). However, its occurrence in the leucine rich and fibroblast associated small PGs (eg. decorin and biglycan) suggests that DS may be important with respect to the cell and ECM regulatory roles played by these PGs. The presence of iduronic acid confers on DS an ability to self aggregate.

Keratan sulfate does not contain an uronic acid, but both its constituent galactose and N-acetylglucosamine may be sulfated at the six position. It occurs in aggregating PGs, usually in domains between the link protein binding sequence an the CS chains. KS is also a constituent of the small PGs fibromodulin (fibroblasts) and perlecan (basement membranes) which, like decorin and biglycan, may participate in cell and matrix regulation (Gallo and Berfield 1996). The physiological role of KS has not been well defined to date.

Heparin is an intracellular GAG with anticoagulant properties. Its extracellular form, HS, is the most complex of the ECM sulfated GAGs with N-sulphate, iduronic acid and *O*-sulfated regions (high charge density regions) of ~12 to 30 disaccharides that are separated by alternating regions of low sulfation (low charge density regions)(Gallo and Berfield 1996). It is common in the basement membranes, and is a widespread component of cell surfaces. The major role of these GAGs is protein and peptide binding (eg. antithrombin III binding). In addition HS has been reported to interact with collagen types I, II, IV and V, fibronectin, laminin, thrombospondin, vitronectin , growth factor binding proteins, cell-adhesion

molecules and cytokines (Gallo and Berfield 1996). Basic FGF selectively binds to penta- or heptasaccharide HS sequences to from a complex with the cell receptor for FGF, mediating the mitogenic response of the cell. Heparan sulfate is also an important component of the ECM of the basement membrane, where it is present in two PGs forms, one of which is perlecan (Timpl 1993; Salmivirta *et al* 1996). In this location the protein core binds to nidogen, and via the HS-GAGs to type IV collagen, fibronectin and laminin.

Proteoglycans and Glycosaminoglycans in Tendons

The PGs constitute less than 1% of adult bovine tendon dry weight, 1-5% in the adult dog, and 2% in the rabbit (Vogel and Heinegard 1985; Okuda *et al.* 1987; Daniel and Mills 1988). Because of the heterogeneity of the protein core of the PGs family much of the connective tissue work investigating their occurrence has focussed on evaluation of different GAGs, using these as indicators of the species of the PGs present in tendon. The quantity and distribution of PGs in tendon differs between those regions subjected only to tensional forces and those where combinations of tensional, frictional and/or compressive forces are sustained (Koob and Vogel 1987b; Okuda *et al* 1987; Birch 1993). In the bovine deep flexor tendon the predominant PGs is decorin, which has been shown to differ in structure from that found in either bone or cartilage (Vogel and Fisher 1986; Evanko and Vogel 1993). However in regions of the tendon subjected to compression or friction, fibrocartilage was identified in which aggrecan and biglycan were elevated compared to the tensile regions (Koob and Vogel 1987b; Okuda *et al* 1987). In the horse decorin, biglycan and fibromodulin have been identified in the digital tendons, as have levels of CS compatible with the presence of aggrecan in the metacarpophalangeal regions of the tendons, but a definitive publication of this work is not yet available (Smith and Webbon 1996).

In the tensional (mid-metacarpal) region of the equine SDFT total sulfated chondroitin sulfate equivalent GAG content is not significantly different between the core and the periphery of the tendon (Birch 1993). In older horses (mean age 14.3 years) the DDFT has a significantly higher total GAG content than the SDFTs (Birch 1993). However,

in a group of unexercised 18-month-old Quarter horses total GAG content was higher in the SDFT, particularly when the proximal metacarpal regions of the two tendons were compared (Bailey *et al* unpublished data). When individual sulfated GAGs were evaluated, differences in the relative proportions of both DS and KS were found between the DDFT and SDFT in one study, and in CS, DS and KS in young unexercised horses (table 2.4). The differences in results between the two studies may the reflect the influences of age, breed and/or exercise history on the ECM composition of the horses evaluated in the two studies, factors which have been determined to be of importance by other workers (Vogel and Heinegard 1985; Vogel and Evanko 1987; Daniel and Mills 1988; Vogel and Thonar 1988; Evanko and Vogel 1993; Patterson-Kane *et al* 1997a&b). Comparisons between the ECM of the tensional and compressive regions of the equine SDFT and DDFT have not been published. However segmental variations in the ECM of the tensile (metacarpal) regions of the SDFT and DDFT, were investigated and higher GAG concentrations approaching the metacarpophalangeal compression zone in the DDFT, and lower GAG concentrations in the mid-tensile zone of the SDFT were found (Bailey *et al* 1996). Similar comparisons have been made in rabbit, dog and cow (Vogel and Heinegard 1985; Koob and Vogel 1987b; Okuda *et al* 1987; Vogel and Evanko 1987; Daniel and Mills 1988; Abrahamsson 1991; Abrahamsson *et al* 1994).

Table 2.4. Proportions of sulfated glycosaminoglycans in equine digital flexor tendons.

Glycosaminoglycan	Proportion of total GAGs in equine tendons (%) HB		Proportion of total GAGs in equine tendons (%) JB	
	SDFT	**DDFT**	**SDFT**	**DDFT**
Chondroitin sulfate	68.5	64.8	46.44 †	43.12 †
Dermatan sulfate	19.2*	10.0*	36.84§	34.82§
Keratan sulfate	12.2*	25.0*	16.70 ‡	22.27 ‡

SDFT = superficial digital flexor tendon. DDFT = deep digital flexor tendon. * Significant differences between SDFT & DDFT ($p < 0.02$*; $p < 0.1$§; $p < 0.01$ †; $p < 0.001$ ‡); n = 6 for both studies; HB = Birch 1993; JB = Bailey *et al* unpublished data.

In the bovine tendon the total amount of GAGs per unit wet weight of tissue is significantly greater (three fold) in the pressure bearing region of the tendon compared to the proximal fibrous region (Vogel and Heinegard 1985). The quantitative and qualitative distributions of PGs in developing bovine tendon are related to the type and distribution of forces to which the tendon is subjected (Evanko and Vogel 1993).

2.6.4 Hyaluronate

Hyaluronate (syn. hyaluronic acid; hyaluronan) consists of repeating units of up to several thousand non-sulfated disaccharide units (*N*-acetylglucosamine + D-glucuronic acid) joined by 1,4 glycosidic bonds. It is the only GAG not ordinarily linked to a core protein, and its chains (up to 10^7 Da in size) are not modified or sulfated (Champe and Harvey 1994; Gallo and Bernfield 1996). It differs from the other GAGs in its synthesis in that it is produced by an enzyme complex associated with the plasma membrane of the cells, through which the growing polysaccharide chain is extruded into the ECM (Prehm 1984; Ghosh *et al* 1992). This results in very large molecules capable of occupying large volumes and binding many cations. The mechanism for controlling HA synthesis is not fully understood, but it has been determined that HA receptors on the cell surface are preferentially stimulated by high molecular weight HA and may be inhibited by low molecular weight HA (Ghosh *et al* 1992). Hyaluronate is not catabolized locally but is circulated via the lymph and degraded by the reticuloendothelial cells, mostly of the liver (Gallo and Bernfield 1996).

Hyaluronate occurs exclusively in the extracellular space (in the ECM and body fluids) and acts as a boundary lubricant and shock absorber of synovial-lined structures, in solution behaving in a viscous manner at low shear rates and as an elastic body at high shear rates (Ghosh *et al* 1992; Gallo and Bernfield 1996; Howard and McIlwraith 1996). It fills the vitreous humor of the eye, but occurs at the highest concentrations in the pericellular region of fibroblasts and other cells of mesenchymal origin (Gallo and Bernfield 1996). In the ECM the large HA chains are highly hydrated, and fold in such a manner that pores or spaces are created which can admit ions and other small molecules. Control of the

concentrations and diffusion rates into and out of the HA complex may affect the activity of these ions and small molecules. In addition, HA may assist in cell aggregation by binding to them via cell surface receptors (Knudson 1993). Several proteins, including the core proteins of the PGs and the link proteins, are able to recognize and bind HA, resulting in the formation of large PGs aggregates in the ECM. The structural importance of HA in the ECM lies in its ability to covalently bind large numbers of PGs molecules in the formation of large PG aggregates such as in aggrecan. In many instances, many PGs monomers are bound to a single hyaluronate molecule in the extracellular space, thereby coalescing into giant PGs aggregates (Abrahamsson 1991). The anionic HA molecule (due to the carboxylic acid residues present in glucuronic acid) is also capable of weak reversible bonding to the positively charged amino acids (lysine, hydroxylysine and arginine) of collagen. However this tendency appears to much lower than that of the sulfonic acid residues of the sulfated GAGs (Nimni and Harkness 1988). The importance of HA in fetal tendon development, where it regulates cell proliferation, migration and differentiation via transmembrane cell surface receptors, has been discussed in section 1.0 (Knudson 1993; Sherman *et al* 1994; McCallion and Ferguson 1996).

2.6.5 Link proteins

The link proteins (LP) are a small family of three closely related glycoproteins which were first discovered in cartilage, and later in other connective tissues (Neame and Parry 1993; Binette *et al* 1994). Recently three LPs (*mw* = 41,000, 43,000 and 46,000) have been isolated from adult equine cartilage and the complimentary DNA sequenced (Dudhia and Platt 1995). They have separate binding domains which facilitate their linkage to PGs and to HA which in turn stabilizes the PGs aggregates (aggrecans and versicans) and protects the HA binding domain of PGs from proteolytic enzymes (Neame and Parry 1993). No other function has been determined for LP. In the absence of LP, PGs aggregates tend to be smaller and less stable. The binding domain on LP for HA has similarities with those of the CD44 and TSG-6 cellular HA receptors. In addition to binding sites, two specific sites for

cleavage of LP by stromeolysin, collagenase, gelatinase and matrilysin (the metalloproteinases) have been identified (Nguyen *et al* 1993). Although LP's have not been specifically identified in equine SDFT's, they are likely to be present in these tissues.

2.6.6 Proteoglycan - Collagen Interactions

The formation and aggregation of collagen into more complex structures and the ageing of these aggregates has been reported to be affected by interactions with both the protein core and the GAG sidechains of PG molecules (Vogel *et al* 1984; Scott 1985; Scott and Haigh 1985; Brown and Vogel 1989; Scott 1990). The PGs and their relationships with collagen types I, II, III and VI appear to be complex and their investigation difficult (Kresse *et al* 1993). The interactions between the two groups of molecules are both direct and indirect, the indirect effects being facilitated by the interaction between PGs bound growth factors, and the cells within the ECM which respond by increasing or decreasing collagen synthesis. The interactions of the smaller PGs , although not fully characterized, are better understood than those of the larger PGs because of their simpler structure. Decorin (PG-II or PG-S2), with its single DS chain, decorates the surface of type I and II collagen fibrils both *in vitro* and *in vivo* (Heinegård *et al* 1988; Kresse 1993). In soft connective tissue decorin occurs regularly and is orthogonally arranged at the bands of type I collagen where it binds to the fibril by its protein core (not the GAG side chains). When decorin is bound to the surface of collagen fibrils in tendon tissue, the lateral assembly of individual triple helices into the forming fibril is delayed, and the final fibril diameter is reduced *in vitro* (Vogel *et al* 1984). This action has been shown to be due to the protein core, and does not occur in response to DS-GAGs alone, or when large PGs are used (Kresse *et al* 1993). This latter finding is not true of all PGs interactions. In the corneal stroma the self association of decorin DS-GAGs (which are of constant length) on opposite collagen fibrils is thought to maintain a constant interfibrillar space and thus contribute to uniformity of corneal structure. In fibroblast cultures is has also been demonstrated that decorin is essential for bridging collagen fibrils and therefore transmitting forces generated by fibroblasts from one cell to

another (Kresse *et al* 1993). The function of biglycan (PGI or PG-S1) is not known, but it interacts with other ECM constituents under suitable conditions *in vitro* (Kresse *et al* 1993).

Fibromodulin, which is a KS linked PGs also has inhibitory effects on collagen fibril formation while lumican, which facilitates the development and maintenance of corneal transparency, is also a KS bearing PG (Kresse *et al* 1993). The differences in their activities are therefore not due to the KS moiety alone, but a result of interaction with the protein core (possibly via their signal peptides), or the protein-KS combination (Scott 1988; Kresse 1993). Fibromodulin, has recently been found to influence collagen fibrillogenesis in vitro, with marked retardation of collagen types I and II (Hedbom and Heinegard 1989). It has considerable homology with the small interstitial proteoglycan decorin (Oldberg *et al* 1989).

Other studies have focussed on the response of collagen to the different GAG moieties alone and have revealed complex responses particularly to CS and DS. Investigations in rat tendon using both cupromeronic blue dye with electron microscopy and biochemical techniques have confirmed that the GAG moieties of the PGs interact electrostatically in a reversible manner with type I collagen. This affinity is dependent upon molecular shape and linear charge density, and provides up to 25% of the stabilization forces in tendon collagen (Nimni and Harkness 1988; Scott 1990). Glycosaminoglycans were not actively incorporated into type I or II collagen fibrils. However CS and DS linked PGs may be incorporated into type I fibrils if present during fibrilogenesis (Scott 1988). It has also been reported that CS and DS accelerate fibre formation during the nucleation phase, but when CS, HS or PGs are added after the nucleation phase, the growth phase of fibrilogenesis is delayed, limiting fibril diameter (Nimni and Harkness 1988; Parry and Craig 1988). It has also been suggested that DS may inhibit the calcification of fibrils (Scott and Haigh 1985).

The basal laminae are continuous thin sheets of ECM that surround many cells, including connective tissue cells. One of the most documented collagen-PG interactions is that between type IV collagen, HS, laminin and entactin, the latter two molecules being basement membrane glycoproteins (Alberts *et al* 1987; Timpl 1993). The HS-GAGs are associated with two different protein cores, one of which is called perlecan. It appears that the glycoprotein nidogen binds to the core protein of perlecan, whereas the HS component

of perlecan mediates PG interactions with laminin, fibronectin and the central region of the type IV collagen triple helix (Timpl 1993). Studies of collateral ligaments have also suggested interactions between type VI collagen and CS (Bray *et al* 1991).

2.6.7 Other Glycoproteins

There are several other structural or connective tissue proteins that contribute to the extracellular matrix of tendon. These include fibronectin, laminin, tenascin, thrombospondin, von-Willebrand factor, nidogen and fibrillin (Birch 1993).

Fibronectin

Fibronectin an adhesive glycoprotein which is distributed throughout most connective tissues, has a high affinity for denatured collagens and is reported to serve as a mediator between the collagen matrix and the interior of tenocytes, facilitating cell adhesion and migration (Potts and Campbell 1994). It exists in a soluble form in blood plasma and is involved in clotting and wound healing, in an insoluble form in the ECM, and as a component of basement membranes where it interacts with cell surfaces (Alberts *et al* 1987; Raghow 1994). The molecule is a 540 kDa dimer of two similar polypeptide chains, with peptide domains for binding fibrin, cell surfaces, collagen, heparin, CS and its integrin receptor $\alpha_5\beta_1$ (the latter being essential for appropriate assembly) (Potts and Campbell 1994).

Laminin

Laminin is a constituent of basement membranes, and therefore is in contact with cells of mesenchymal and epithelial origin. It consists of three very long polypeptide chains arranged in a cross-like shape (Alberts *et al* 1987). Like fibronectin, it has binding domains for type IV collagen, HS and cells, but the responses of cells to these may be either stimulatory or inhibitory (Raghow 1994). Its activities include promotion of cell

proliferation, attachment and chemotaxis, inhibition or stimulation of angiogenesis, and induction of collagenase IV and tyrosine hydroxylase enzymes. Both the entire molecule and its subfragments have demonstrated biological activity.

Cartilage Oligomeric Matrix Protein

Recently cartilage oligomeric matrix protein (COMP) has been demonstrated to be present in adult, but not neonatal, bovine and equine digital flexor tendons (Smith and Webbon 1996). It is present at high levels all regions of the equine flexor tendons (~3% dry weight), and occurs in higher concentrations in the SDFT (especially in the mid-metacarpal region) than the DDFT. The precise function of COMP is not known, but there is evidence that it may bind to other molecules, and may have a role as a transfer protein, similar to ceruloplasmin (Stanescu *et al* 1994). Other workers have suggested that based on its distribution in tendon and its variation with age, COMP is a structural protein synthesized in response to loading (Smith and Webbon 1996). It has been proposed as a marker for determining the response of equine tendons to exercise and disease.

2.6.8 Regulation of Matrix Synthesis and Catabolism

The complexity of the extracellular matrix suggests regulatory mechanisms which are multifarious in nature. Just as a comprehensive understanding of the structure of the ECM of tendons is not yet available, the regulation of matrix synthesis and catabolism of connective tissues is poorly understood (McIlwraith 1996). Given the complexity of cell signalling (autocrine, paracrine and systemic) within the animal, and intermolecular interactions within the ECM, it is unlikely to be fully understood in the near future. Essentially, regulatory responses are initiated by receptor activation on the cell surface (by cytokines, mechanical or electrical stimuli, or other signalling molecules), triggering either a biochemical and/or a mechanical response in the case of the cells of the tendons, ligaments and other connective tissues. Different aspects of matrix synthesis and regulation as they

relate to cell-cell, cell-matrix and matrix molecule-ECM interactions have been introduced in previous sections of this dissertation, and these all contribute to an understanding of the processes and molecules involved in regulation of the ECM.

The role of cell-cytokine contributions to this process of tendon homeostasis is poorly understood, as is the regulation of homeostasis of normal tendon tissue. In cartilage inteleukin 1 (IL-1), interleukin 6 (IL-6), inteleukin 8 (IL-8), tumor necrosis factor (TNF), insulin-like growth factor (IGF) and transforming growth factor beta (TGF-ß) have been demonstrated as necessary for intercellular communication and the regulation of synthetic and catabolic pathways (Platt 1996). Although there is little information available on the effects of cytokines on tendon tissue, it is expected that some of the responses observed in cartilage will be discovered in other connective tissues. Recently IGF-I was shown to have an anabolic effects on collagen metabolism in the equine SDFT (Murphy and Nixon 1997). The effects of ageing and exercise on different aspects of the tendon matrix development and maturation have also been introduced in previous sections, and appear to have significant bearing on the biochemical composition and molecular structure of the ECM (Koob and Vogel 1987b; Patterson-Kane 1997a&b).

Several enzymes have been identified in cartilage and tendon tissue which are responsible for the extracellular catabolism of the connective tissue ECM during disease, where they are essential for repair and tissue remodelling, and during normal homeostasis (Gailit and Clark 1994; McIlwraith 1996). They include the metalloproteinases and the tissue inhibitors of metalloproteinases (TIMPS), as well as serine, cysteine and aspartic proteinases.

Metalloproteinases

The metalloproteinases (MMPs) are a group of proteolytic enzymes characterized by their requirement for Zn^{2+} at their active site (McIlwraith 1996). They are present extracellularly at high concentrations during the inflammatory and early reparative phases of wound healing, decreasing as the wound remodels and matures (Gailit and Clark 1994). All MMPs are secreted from fibroblasts, synovial cells, osteoblasts, chondrocytes, endothelial

cells, macrophages, and keratinocytes in an inactive zymogen form which is activated by other MMPs, or serine proteases such as plasmin, trypsin or neurophil elastase (Mignatti *et al* 1996). The mechanism of their activation during normal tissue homeostasis is not understood. Each of the MMPs act on one or more specific molecular substrates at specific sites. Link protein for example has two domains at which different MMPs cleave the protein (Nguyen *et al* 1993). Stromeolysin 2 and gelatinase A cut LP at both these sites (creating three fragments); collagenase, gelatinase B and stromeolysin 1 cut at one site; matrilysin cuts at the other site. Many of the protein cores of the PGs and the individual collagen molecules also have specific sites at which the MMPs act. The best characterized MMPs are the collagenases. Interstitial or tissue collagenase (MMP-1) is specific for collagen and cleaves all three chains of the molecule at one specific site between a glycine and isoleucine residue of the α_1(I) chain of collagen types I,II and III (McIlwraith 1996). It also cleaves collagen types VII, VIII and X, but not basement membranes or types IX and XI. The other major MMPs thought to be important in connective tissue ECM are stromeolysin 1 and 2 (MMP-3 and MMP-10). Table 2.6 summarizes the substrate activity of the MMPs.

The MMPs are regulated largely by a family of tissue derived inhibitors of the metalloptoeinases (TIMPs), three of which have been identified and labeled as TIMP (or TIMP-1), TIMP-2 and TIMP-3 (Mignatti *et al* 1996). The TIMPs bind covalently and irreversibly to inhibit the catalytic activity of MMPs but not other proteases, and have also been reported to have growth promoting activity for many cell types (Hayakawa *et al* 1992). TIMP-1 is a glycoprotein which inhibits all mammalian MMPs (but not collagenase of bacterial origin) and acts only on the active enzymes and not the zymogens (except for the 92 kDa progelatinase). Due to the presence of six disulfide bonds and their molecular configuration, the TIMPs are highly resistant to extremes of pH and temperature. The activity of TIMP-2 is directed mostly at the 72k Da gelatinase, and although similar in structure to TIMP-1, it is not glycosylated. The activity of the recently discovered TIMP-3 is not known but this MMP inhibitor is localized predominantly within the ECM (Mignatti *et al* 1996).

Table 2.5: Metalloproteinases associated with tissue remodelling.

Metalloproteinase	Substrates
Tissue collagenase	
MMP-1†	Collagen types I, II, III, VII & X, LP
Gelatinases	Denatured type II, gelatin, elastin, entactin
A (72 kDa) MMP-2†	Collagen types X & XI , LP,
B (92 kDa) MMP-9†	Collagen types IV & V, LP
Stromeolysins	
(1) MMP-3†	Procollagens, collagen types IX & XI, elastin, LP, fibronectin, decorin, aggrecan, activates procollagenase
(2) MMP-10‡	Fibronectin, LP, activates procollagenase
Neutrophil collagenase	
MMP-8‡	Proteoglycans, gelatins, fibronectin
Macrophage metalloelastase	Elastin, collagen type IV
Matrilysin	Collagen type IV, elastin, laminin, entactin, elastin, LP

LP = link protein; * gelatins = denatured collagens; †inhibited by TIMP-1 and TIMP-2; ‡inhibited by TIMP-1. (Gailit and Clark 1994; McIlwraith 1996; Mignatti *et al* 1996)

Serine Proteinases

The serine proteinases are the other enzyme group that plays a major role in physiological and pathological tissue remodelling (Mignatti *et al* 1996). The enzymes in this group include plasmin, tissue plasminogen activator (tPA), urokinase (uPA), neutrophil elastase, cathepsin G and plasma kallikrein. The PAs convert the zymogen plasminogen to plasmin which, in turn has a broad substrate activity which includes fibronectin, laminin, gelatins and the core protein of PGs (Mignatti *et al* 1996). In addition to its catabolic activity, plasmin mediates the release of basic FGF from cell surfaces and the ECM. Plasmin

is also an activator of the MMPs, but is inhibited by the serum protein α_2-antiplasmin. Their activity is regulated by PA inhibitors (PAI-1, PAI-2 and PAI-3) and protease nexins, which can bind and inactivate the activators (McIlwraith 1996). Interleukin-1 has been demonstrated to stimulate the production of tPA but not uPA. Elastase and cathepsin G are produced by neutrophils and are potent degraders of PG, and inhibitors of PG synthesis. Both are inhibited by α_1-proteinase inhibitor (α_1-PI).

Cysteine Proteinases

Cathepsins B, L and H are lysosomal proteinases, with cathepsin having the broadest pH range for activity (McIlwraith 1996). Cathepsins B and L cleave the end terminal peptides of collagen that contain the intermolecular and intramolecular cross-links. In addition, cathepsin B cleaves the HA binding domain on PGs, and degrades and fragments the GAG attachment region. Cathepsin L causes more extensive degradation than B, but both cleave all three LPs at multiple sites. As with the MMPs, the cathepsins have active and inactive forms. They may also be stimulated by IL-1 and tumor necrosis factor-α (TNF-α), but are inhibited by molecules called cystatins. These are secreted by the same cells which secrete the cathepsins - synoviocytes and chondrocytes, and imbalances between the two may result in orthopedic disease. The other member of this group of proteinases are the calpains which are Ca^{2+} dependent enzymes in the cell cytosol capable of degrading monomer and aggregated PGs. Their corresponding inhibitors are the calpastatins.

Aspartic Proteinases

Cathepsin D requires an aspartate residue in its structure to facilitate its catabolic action during inflammation and rapid ECM breakdown (McIlwraith 1996). It is secreted into the extracellular space by macrophages and connective tissue cells where it degrades PGs by cleaving the HA binding domain on PGs adjacent to the sulfated GAG attachment region.

2.6.9 Segmental Variation in the Equine Digital Flexor Tendons

Histologic Variation

Until recently, there were very few published works which had examined the degree of histologic or biochemical variation due to age or location along the length of the equine digital flexor tendons (Otomo 1973; Webbon 1978). Early histologic comparisons have been made within the normal SDFT between the tendon at the musculotendinous junction and within the carpal sheath, the metacarpal (extrasynovial) region, the tendon within the digital sheath, and the insertion of the tendon into the first and second phalanges (Webbon 1978). The surface of the musculotendinous junction and within the carpal sheath is covered by a layer of densely staining nuclei, with the proximal fibres forming an intermeshing network which become longitudinally oriented more distally. The tendon of the metacarpal region is surrounded by loosely arranged tissue or the paratenon, and has wavy, eosinophilic fibres with reduced cellularity compared to the intrasynovial region. The cells are arranged in longitudinal columns. Chondroid metaplasia occurs occasionally in the metacarpal region (particularly in older horses) and in some regions the cells are arranged irregularly, resulting in 'acellular' areas. Tendon within the digital sheath does not have the low cell density observed in the metacarpal region and there is a well developed vascular zone below the synovial layer. In the region of periosteal insertion more chondroid cells are observed.

Recently cell density was determined in a sequential manner by morphometric techniques along different zones of the tensile equine SDFT and compared to the DDFT (Bailey *et al* unpublished data). The cell density of the endotendon was counted separately, and although there was a tendency for a higher density in the SDFT, differences among the zones in either tendon were not significant. The cell density of the remaining matrix was significantly higher in the middle of the SDFT, and increased significantly from proximal to distal in the DDFT. The mean cell density of the SDFT was approximately double that of the DDFT. Although all the horses were 18-month- old Quarter horses raised on the same farm, there were significant differences among the horses for the parameters measured.

Biochemical Variation

Regional differences in cellular proliferation and matrix synthesis have been identified in many other species, reflecting differences in the mechanical requirements of different tendons and different zones each tendon (table 2.5) (Kain *et al* 1988; Abrahamsson *et al* 1994). In a parallel study to the histologic study by Bailey *et al*, biochemical parameters were determined and compared in a similar manner (Riley *et al* 1995; Bailey *et al* 1996). In the DDFT, uronic acid (UA) concentrations (an indicator of CS, DS, HS and HA but not KS) were significantly higher for the distal zones as the compression region of the tendon was approached. In the SDFT the UA concentration was significantly lower in the middle zones, a finding compatible with the findings in other species (Vogel and Heinegard 1985; Okuda *et al* 1987; Daniel and Mills 1988; Vogel and Thonar 1988; Abrahamsson *et al* 1994). When CS, DS and KS concentrations were individually determined there was a significantly higher concentration of CS in the SDFT than in the DDFT, a tendency for higher DS concentrations in the SDFT than in the DDFT ($p < 0.1$), but a significantly higher KS concentration in the DDFT than the SDFT (Bailey *et al* 1996). A segmental comparison along the tensile SDFT did not indicate significant differences in CS, DS or KS concentrations. However in the DDFT, CS levels were significantly higher in the distal zone (those approaching the metacarpophalangeal joint), but DS and KS levels were significantly higher in the proximal zones. In addition to these findings, collagen concentration did not vary segmentally in either tendon or between the SDFT and DDFT. Recently, these differences in total GAG content in the SDFT have recently been parallelled by a study which demonstrated segmental differences in the mechanical properties of the tendon (Crevier *et al* 1996).

Table 2.6. Proportion of individual glycosaminoglycans in digital flexor tendons.

Specie	Region	Predominant Proteoglycan	Predominant GAG chain	Other GAG chains
Rabbit	Compression	high MW	CS	KS
	Tension	low MW	DS	
Canine	Compression	Not reported	CS	DS
	Tension	Not reported	DS	HA
Bovine	Compression	high MW	CS	KS
	Tension	low MW	DS	CS

CS = chondroitin sulfate; DS = dermatan sulfate; KS = keratan sulfate; HA = hyaluronate ;(Vogel and Heinegard 1985; Okuda *et al* 1987; Daniel and Mills 1988; Vogel and Thonar 1988; Abrahamsson *et al* 1994)

2.7 Tendon Healing

2.7.1 Extrinsic Versus Intrinsic Healing

The healing of the SDFT has been claimed to occur either by the migration of cells and vasculature from surrounding peritendinous tissues (extrinsic healing) or by outward growth from the cut or damaged ends of the tendon (intrinsic healing). There is considerable controversy in the literature over the relative importance of contributions from intratendinous and peritendinous blood supply and cellular proliferation to tendon healing. However, there is considerable evidence for both contributions to healing and, depending upon the type of injury that occurs and whether or not the injury is intrasynovial or extrasynovial, it has been suggested that a combination of intrinsic and extrinsic factors is necessary for optimal healing (Graham *et al* 1984; Lundborg and Rank 1987; Kain *et al* 1988; Abrahamsson 1991; Abrahamsson *et al* 1994; Pool 1996).

The concept of extrinsic healing has been used as one of the rationales for surgical tendon splitting in chronic cases of equine SDFT tendinitis (Asheim and Knudsen 1967). The formation of adhesions between the injured tendon and surrounding tissues, and the relative

hypocellularity of tendons have also been interpreted as evidence of extrinsic contributions to healing (Potenza 1962; Peacock 1984). In lacerations of the equine flexor tendons healing is dominated by extrinsic responses in the early stages of repair, and intrinsic healing is minimal due to disruption of the intratendinous vasculature and cells and ECM at the wound margins. In cases where contamination is minimal, it is recommended that the tendon ends be reapposed, and that suture techniques which minimize damage to the intratendinous vasculature are used - both aimed at supporting intratendinous healing and minimizing scar formation (Bertone *et al* 1990; Easley *et al* 1990; Jann *et al* 1991; Mahshadi and Amis 1991; Jann *et al* 1992a; Jann *et al* 1992b; Watkins 1992b; Montgomery *et al* 1994). Lacerations involving the tendon sheath, those involving other connective tissue support structures, and those that have a prolonged interval between injury and treatment carry a poorer prognosis (Wagner and Shires 1986; Spurlock 1989). Where the tendon sheath is involved both pathways for nutrition -perfusion and diffusion, are disrupted. It has been suggested that diffusion is an important nutritive pathway in these areas, and may account for the poorer prognosis (Smith and Webbon 1996). In man, where intrasynovial tendon injury and surgery is common, extrinsic healing is considered particularly undesirable when it leads to the formation of adhesions. Therefore considerable effort has been put into the careful reconstruction of the injured tendon, pharmacological control of adhesions, and early mobilization of the tendon to minimize adhesions and optimize intrinsic healing (Bora 1987; Gelberman and Manske 1987; Kutz 1987; Lundborg and Rank 1987; Michon 1987).

There is also strong support for stimulating and optimizing the intrinsic healing capacity of the flexor tendons (Lundborg and Rank 1987; Schepel 1987; Abrahamsson 1991; Abrahamsson *et al* 1994). Cells from the epitendon and endotendon proliferate to repair the tendon defect (Lundborg and Rank 1987; Riley *et al* 1996). Studies in the rabbit found that tendon segments introduced into synovial compartments survived without developing adhesions (Furlow 1976; McDowell and Snyder 1977).

In studies of equine SDFT tendinitis, Webbon (1973) found evidence of cellular proliferation in both the endotendon and peritendinous regions of the injured equine SDFT (suggesting intrinsic and extrinsic contributions to healing) *in vivo*. In acute lesions,

examined a few days after onset of clinical signs, there is pyknosis and death of tenocytes in the core region, haemorrhage, disruption and disintegration of collagen fibres as well as oedema (Norberg *et al* 1967; Stromberg 1971). This may be accompanied by extensive subcutaneous and peritendinous haemorrhage (Webbon 1977). Within 7-21 days of acute injury, the infiltration of mesenchymal cells is increased, with a gradual increase in the number of vessels growing into the lesion from the periphery (Stromberg 1971). Whether the origin of these vessels was peritendinous or endotendinous was not clear. However, intrinsic factors have been most commonly implicated including vascular changes within the middle metacarpal region of the SDFT, and changes in the core temperature of the tendon (Asheim 1964; Stromberg and Tufvesson 1969; Stromberg 1971; Wilson and Goodship 1991). The support for extrinsic healing in these lesions when they are not extensive enough to involve the epitendon is scant, and ultrasonographic studies have not demonstrated changes in echogenicity which would be consistent with extrinsically based remodelling (Genovese *et al* 1996). Both *in vitro* and *in vivo* studies in the horse and other species have confirmed the intrinsic capacity of flexor tendons for repair, and support the development of treatment methods aimed at enhancement of intrinsic tendon healing (Gelberman *et al* 1984; Manske *et al* 1984; Manske *et al* 1985; Mass and Tuel 1990; Mass and Tuel 1991; Kraus-Hansen *et al* 1992). Several recent publications have reported research focussed on the manipulation of the intrinsic healing capacity of the equine SDFT (Dahlgren *et al* 1996; Dahlgren *et al* 1997; Riley *et al* 1996; Murphy and Nixon 1997).

2.7.2 Tendon Healing *In Vivo*

With supportive treatment and time, the injured tendon heals with a scar but usually remains enlarged and slightly sensitive to firm palpation. Experimental results and clinical experience suggest that healing of partial tendon ruptures may take eight to twelve months before the structure approaches normal function (Williams *et al* 1984; Nixon 1990b). The sequence of events following tendon injury is similar to that which occurs in other tissues: inflammation (fluid and cellular exudate); repair (fibroplasia); and remodelling (organization

and maturation) (Peacock 1984; Williams *et al* 1984).

1) Inflammation (days 1-5). Serosanguineous fluid and cells accumulate resulting in oedema of the peritendinous tissues and tendon stumps. Fibrin, platelets and red blood cells bridge the site with a clot. Small numbers of irregularly shaped fibroblasts may be seen after two days, with a maximal rate of proliferation attained by day 7 (Chvapil 1996; Davis 1996).

2) Repair (days 6-28). This phase consists of clot organization, cellular proliferation and matrix synthesis. Fibroblast-like cells (possibly type II tenocytes) from the epitendon and endotendon proliferate and migrate into the site along the network of fibrin. Necrotic cells as well as damaged and undamaged ECM in the locale of the injury are phagocytosed by macrophages and denatured both intracellularly and extracellularly by MMPs, serine proteases and other degradative enzymes. Coincident with the migrating fibroblasts (tenoblasts) there is an increase in the amount of fibronectin in the wound bed (Williams *et al* 1984). This is present throughout the new matrix, in the walls of new capillaries and in areas of unresolved haemorrhage and cellular debris and it preferentially binds denatured or partially denatured collagens. As fibroplasia occurs, endothelial buds penetrate into the injured region by degrading the fibrin network and arterioles are found in the tendon periphery after ten days (Abrahamsson 1991). Type III collagen synthesis predominates, and is distributed in a loose reticulum throughout the matrix one week after injury (Williams *et al* 1984). Type I collagen is also present but is predominantly pericellular, as are types IV and V (Watkins 1985a). The rates of synthesis of PG and GAG increase in tandem with collagen synthesis but taper off more rapidly than collagen by day 28 (Chvapil 1996).

3) Remodelling (day 17-several years). This phase consists of organization and maturation of the granulation tissue filling the wound bed. The collagen is altered from a mostly random pattern of deposition for the first weeks, to a more oriented deposition after day 28 (Chvapil 1996). The proliferating epitendon tissue forms a bulbous callus (Abrahamsson 1991). The fibroblasts become longitudinally oriented and the thicker type I collagen fibres increase in density and number compared to type III fibres (Williams *et al* 1980; Williams *et al* 1984). Elastin fibres are also present and the amount of fibronectin decreases with time. The scar gradually matures but is more cellular and less uniform in

composition than normal tendon with type III collagen persisting for months or even years (Williams *et al* 1984). The persistence of type III collagen affects formation of collagen type I fibrils and lowers mean fibril diameter thereby decreasing the ultimate tensile strength of the tendon. Inherent in the maturation process is an increase in cross-linking which commences between two to three weeks post injury and peaks at three to four weeks (Chvapil 1996; Davis 1996). During this time the tensile strength of the scar increases markedly with a more gradual increase after six to eight weeks.

During tendon healing *in vivo*, collagen synthesis slowly increases over six weeks whereas non-collagen proteins and total protein synthesis peak and plateau at approximately day ten (Kain *et al* 1988; Abrahamsson 1991). Total collagen content and the percentage of collagen synthesized as a proportion of total protein produced decreases. In the healing equine SDFT 30% of all collagen synthesized is type III, compared to 10% in the rabbit and dog (Gelberman *et al* 1984, Williams *et al* 1984).

2.7.3 The Study of Tendon Healing *In Vitro*

Embryonic and adult tendon tissues have been cultured from the chicken, man, rabbit, horse, dog and monkey (Silver *et al* 1983; Gelberman *et al* 1984; Mass and Tuel 1990; Mass and Tuel 1991; Abrahamsson 1991; Riley *et al* 1996). Serum or plasma is necessary to stimulate cell proliferation and migration during *in vitro* tendon healing (Gelberman *et al* 1984; Manske *et al* 1984). Fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) stimulate tendon fibroblast proliferation in explant cultures of rat tail tendon with or without the supplementation of media with serum (Stein 1985). Studies using explant cultures of rabbit flexor tendons indicate that insulin-like growth factor-I (IGF-I) has a potent anabolic effect on protein synthesis and cell proliferation in serum-free and supplemented media (Abrahamsson *et al* 1991a,b). The results of these studies suggest that serum or tissue derived growth and mitogenic factors may be of importance in tendon healing and may be useful for treatment of injured tendons *in vivo*.

During the initial two weeks of tendon culture, epitendon cells proliferate and

encapsulate the cultured segments (Gelberman *et al* 1984). Collagen is synthesized by epitendon cells within the first week of culture, and by the endotendon after two weeks of incubation. DNA and hydroxyproline synthesis also increase in vitro (Abrahamsson 1991). Equine SDFT explants have endcaps of new tissue and an irregular contour within two to four weeks (Riley *et al* 1996; Murphy and Nixon 1997). Cell proliferation, differentiation, migration and collagen synthesis occur in all the species listed above, but at different rates (Gelberman *et al* 1984).

Short and long term cell and explant culture techniques have been reported for the equine SDFT (Silver *et al* 1983; Dahlgren *et al* 1996; Dahlgren *et al* 1997; Riley *et al* 1996; Murphy and Nixon 1997). Tendon healing *in vitro* has many similarities with healing in vivo, with the exception that it proceeds at a slower rate and without systemic regulation (Gelberman *et al* 1984; Manske *et al* 1984). Explant cultures facilitate the study of tendon healing as well as collagen-cell and collagen-proteoglycan interactions. It does not permit study of the effect of systemic homeostatic mechanisms on tendon repair, and limits the study of cell-cell interactions, other than those among the tenocytes themselves.

2.8 Pharmacological Agents and Tendon Healing

2.8.1 General Principles for the Management of Tendon Injury

There are many conditions affecting the equine SDFT and its associated structures but lacerations and degenerative tendinitis are the most common lesions reported and the discussion of pharmacological treatment of tendons will be limited to their possible use for these conditions. Although both conditions follow a course of injury, inflammation, repair and maturation of the scar, the timing of these events differs for the two types of injury.

There have been many therapeutic regimes applied in an attempt to influence the duration of the inflammatory, debridement, reparative and maturation phases of tendon healing and to improve the quality of the resultant scar tissue. However, the results of treatment of SDFT tendinitis are frequently unsatisfactory due to a high rate of reinjury

following return to athletic activity (Norberg *et al* 1967; Watkins 1992a). Tendon lacerations have also been reported to have guarded prognosis (Foland *et al* 1991). The aim of initial treatment following acute injury is to reduce and minimize inflammation and prevent further injury. Therapy for the acute inflammatory phase of injury usually consists of cold therapy, complete rest, topical anti-inflammatory application (in the case of tendinitis), antiphlogistic wraps, non-steroidal anti-inflammatory drugs (NSAIDS) and firm pressure bandages (Webbon 1973; Nixon 1990a). In severe cases, immobilization in a cast for 1-2 weeks or splinted support has been suggested (Rapp *et al* 1991; Rapp *et al* 1992).

Once the acute inflammation has subsided (usually 3-5 days), physical therapy following injury or surgery has been identified as an important part of the rehabilitation program for the patient. The program used may affect scar formation, remodelling, and adhesion formation (Gelberman *et al* 1983). Passive mobilization and massage of the tendon may commence within a few days after injury and has been demonstrated to increase collagen synthesis and inhibit the activity of collagenase (Amiel and Kleiner 1988; Gaughan *et al* 1991; Rapp *et al* 1992; Kubota *et al* 1996; Nabeshima *et al* 1996). Rest alone for up to twelve months has been recommended but many clinicians favour stall rest with a graduated program of controlled passive or mild exercise for one to twelve months depending upon the degree and response of the injury (Nixon 1990a; Rapp *et al* 1991; Watkins 1992a; Gillis *et al* 1996). Passive exercise may take the form of swimming in a suitable facility (Nixon 1990a). Following ultrasonographic confirmation of satisfactory tendon healing, horses may be jogged and gradually returned to training (Watkins 1992a; Gillis *et al* 1996).

The use of pharmacological agents, both experimentally and clinically, for the treatment of SDFT tendinitis has failed to reduce adhesion formation, promote more rapid tendon healing or reduce scar formation in either man or animals (Gaughan *et al* 1991). Agents which have been used include corticosteroids, antihistamines, lathyrogen, ß-aminopropionitrile, orgotein, exogenous collagen, dimethyl sulphoxide, hyaluronidase, sodium hyaluronate (NaHA) and polysulfated glycosaminoglycans (PSGAG). Recent attention has been focussed on the use of NaHA (Legend® or Hyonate®), PSGAG (Adequan®) and ß-aminopropionitrile (Nixon and Gaughan 1989; Schmidt 1989; Goodship

et al 1992; Marr *et al* 1993; Chvapil 1996; Dahlgren *et al* 1996; Davis 1996; Ordidge 1996). In contrast to human digital tendons where most research has been directed at pharmacological control of adhesions, most of the therapeutic regimes examined for equine tendon injury have focussed on treating inflammation or modulating the rate and type of repair in the tendon (Bora 1987; Gaughan *et al* 1991; Marr *et al* 1993) .

2.8.2 Anti-inflammatory Agents

Corticosteroids

The use of corticosteroids for treatment of inflammatory conditions of the connective tissues has had a long and controversial history. This is in part due to the small amount of information available about the effect of these drugs on the fibroblasts and the ECM, and partly due to some poorly founded but highly publicised assumptions that first entered the equine literature in the late 1960's (O'Connor 1968; Trotter 1996b). Corticosteroids consist of three 6-carbon rings and a 5-carbon ring, with activity determined by the presence of a hydroxyl group at the C-11 position (Trotter 1996). Molecular substitutions at various sites has resulted in the production of synthetic corticosteroids with improved anti-inflammatory activity, decreased mineralocorticoid activity and altered solubility. Phosphate and succinate esters are water soluble and good for parenteral use, but the acetate and acetonide esters are more lipid soluble thus delaying their absorption following intra-articular administration, and prolonging their duration of action. Triamcinolone hexacetonide has the longest duration of action of corticosteroids currently used in the horse (Trotter 1996). To date the only corticosteroid that has been critically evaluated in the equine SDFT is methylprednisolone acetate (MPA) which is an intermediate to long-acting drug in the horse (Pool 1996; Trotter 1996). There are also a few anecdotal reports of its use by clinicians in cases of tendinitis, tenosynovitis and desmitis of the fetlock annular ligament (Goodrich and White 1997).

The reported range of activities of corticosteroids is diverse and will be only briefly summarized as they pertain to the degradation and synthesis of connective tissue ECM.

Corticosteroids pass into the cell cytoplasm and bind with steroid specific receptors, resulting in a conformational change in the receptor complex (Trotter 1996). The complex then is able to reversibly bind to the nuclear chromatin associated with glucocorticoid responsive genes and to modulate the transcription of mRNA from these genes. The anti-inflammatory effects of corticosteroids are potent, influencing cellular (leukocyte movement) and humoral aspects of the process. These drugs inhibit leukocyte migration into the site of inflammation, increase bone marrow production of neutrophils and prolong the half-life of neutrophils; all of which contribute to neutrophilic leukocytosis. The reduction in macrophage accumulation at the site of injury may inhibit chemotaxis and adherence of neutrophils to the vascular endothelium, and later, of fibroblasts into the wound during the repair phase. The most notable humoral affect is that of inhibiting the synthesis of prostaglandin from arachidonic acid, thus minimizing the effects of the pro-inflammatory products of cyclooxygenase and lipooxygenase pathways (Higgins and Lees 1984).

In connective tissues triamcinolone has been shown to decrease the expression of collagenase and TIMP mRNA in synovial tissue from human rheumatoid arthritis patients (Firestein *et al* 1991). However corticosteriods did not suppress stromeolysin synthesis by equine synovial cells *in vitro* (May *et al* 1988). In equine synovial fluid PG concentration is elevated after either methylprednisolone or betamethasone administration and although stromeolysin induced cleavage of PG aggregates was suggested as the reason, this has not been substantiated as the mechanism of action (Trotter 1996). Corticosteroid suppress HA (but not sulfated GAG) synthesis in human skin fibroblast and canine synovial cultures. However, *in vivo* studies in the horse showed increased HA concentrations in the synovial fluid following intra-articular corticosteroid administration (Trotter 1996). The effect on cartilage depends upon the type and dose of drug used as well as the species of animal and the design of the trial (for excellent review see Trotter 1996).

In the only published equine SDFT studies to date, MPA was first injected by an intratendinous route along the tensile length of the normal SDFT and compared to carrier injected and saline controls (Pool 1996). Significant histopathologic findings were not present in the control groups between 3 to 14 days post-injection. However at day 3 in the

MPA group there was vascular thrombosis and acute focal necrosis of the small blood vessel walls of the endotendon, epitenon and paratenon. Adjacent to the crystalline deposits of MPA, most tenocytes were dead. Two weeks after MPA injection there was a focal inflammatory process characterized by the presence of macrophages, some containing engulfed crystals of MPA. The damage to vessels, tenocytes and macrophages may be due to the physical form of the drug, rather than a pharmacological response. In a parallel study, tendons with SDFT tendinitis that had been injected with corticosteroids were examined at autopsy (Pool 1996). Many of the microscopic abnormalities in these tendons did not differ significantly from those found in untreated horses with tendinitis. However in several tendons treated with corticosteroids, there were increased numbers of hyalinized and mineralized fibres and linear mineralized amorphous foci compared to untreated horses. Although hyalinized collagen frequently mineralizes in aged horses and in cases of untreated tendinitis the authors concluded that the crystals of MPA may create nidus for the initiation of dystrophic mineralization (Webbon 1978; Pool 1996; Smith and Webbon 1996). Local injection of an injured tendon with MPA may increase the amount of local damage present, accelerate or potentiate dystrophic calcification and delay the reparative phase of tendon healing (Pool 1996).

In man the use of corticosteroids (triamcinolone acetonide) has been recommended only for difficult cases for adhesion prevention following surgery in order to preserve the gliding function of intrasynovial tendons following tenolysis (Strickland 1987). Similar recommendations have been made for its use in the treatment of equine cases of tenosynovitis, either alone or in combination with NaHA for the prevention of adhesion formation and reducing inflammation (Nixon and Gaughan 1989; Gaughan *et al* 1991; Nixon 1996). The use of corticosteroids in severe cases of equine fetlock annular ligament desmitis was recently reported, and appreciable side effects were not observed clinically (Goodrich and White 1997).

The non-steroidal anti-inflammatory drugs (NSAIDs) are agents which inhibit the conversion of arachidonate to prostaglandins and thromboxane (May and Lees 1996). Phenylbutazone and flunixin megulamine are the NSAIDs most commonly used in the horse and are competitive antagonists of cyclooxygenase. In contrast with aspirin which irreversibly antagonizes by acetylation and deactivation of cyclooxygenase, their activity depends upon their continued presence in the tissues (May *et al* 1984). Although used in tendon injury for their general ability to reduce inflammation and associated pain, the NSAIDs have side effects which include increased bleeding time due to decreased platelet aggregation (aspirin only), gastrointestinal ulceration, renal papillary necrosis and blood dyscrasias. The NSAIDs appear to stimulate synthesis of ECM moleculaes by cartilage in addition to their effects on the inflammatory cascade (Palmer and Bertone 1994; May and Lees 1996). Sodium salicylate and aspirin have been reported to inhibit proteoglycan synthesis, with a more pronounced effect with salicylate in osteoarthritic cartilage (May and Lees 1996). Phenylbutazone does not appear to affect equine articular cartilage anabolism (Cambridge and Lees unpublished data). It is possible, but not proven, that some NSAIDs may also exert some effects on matrix synthesis in normal and/or diseased tendons.

2.8.3 Sodium Hyaluronate

Commercial preparations of sodium hyaluronate are commonly used for the treatment of equine orthopaedic disease and like the naturally occurring HA of the ECM, consist of chains of disaccharide subunits (*N*-acetylglucosamine linked to D-glucuronic acid by 1-3 glycosidic bonds) joined by 1-4 glycosidic bonds but vary in their biological source (see section 2.6.4). In normal equine horse synovial fluid the average molecular mass of HA was 1.5×10^6 daltons (range 0.5 to 3.0×10^6 daltons) in one study, and 2.5×10^6 daltons (range 2 to 3×10^6 daltons) in another study (Tew 1984; Tulamo 1994).

Table 2.7: Summary of major sodium hyaluronate products available for equine use.

Product	NaHA Concentration (mg/ml)	Molecular Weight	Manufacturer
Hylartin®	8.9	2652703	Kabi Pharmacia, AB, Sweden
Hyvisc®	9.2	2449000	Anika Research, Inc.
Equron®	5.2	766500	Solvay Animal Health, Inc.
Map 5®	9.5	757200	Vetrepharm Inc.
Hyalovet®	9.8	605500	Fort Dodge Laboratories, Inc
Legend® i/a	10.3	361900	Bayer Corporation †
Legend® i/v	10.9	321600	Bayer Corporation †
Synacid®	9.2	82240	Schering Plough Animal Health Corporation

†Also called Hyonate®, Bayer Canada. (Ghosh *et al* 1992; Uden and Lavoie 1997).

The commercially available NaHA products for equine use in North America vary widely in protein concentration, intrinsic viscosity and molecular weight (Uden and Lavoie 1997). In a comparative study, viscosity was found to increase with increased molecular weight, with the highest viscosities reported for Hylartin® and Hyvisc®(table 2.7) (Uden and Lavoie 1997).

The reported and potential actions of exogenous NaHA are many, including effects on inflammation and on ECM catabolism and synthesis. Inflammatory cells are affected, with HMW-HA reported to prevent neutrophil migration by binding and disrupting chemoattractant gradients necessary for cell locomotion, in a dose dependent manner (ie. a steric effect) (Balazs 1985). Solutions of high viscosity or HMW-HA also inhibit the motility and phagocytic activity of macrophages in a dose dependent manner. Although steric hindrance was thought responsible for these inhibitory effects, it is now believed that they are mediated by the interaction of HA with CD44 receptors on the cells (Tamoto *et al* 1993). This finding is important since it infers that effective CD44 binding and activation requires

a minimum chain length of HA, and that smaller chains may act to competitively block the cellular responses to HA (Knudson 1993). *In vivo*, HMW-HA inhibits prostaglandin (PGE_2) levels in synovial fluid, further moderating the inflammatory response (Punzi *et al* 1989).

The cell surfaces of synoviocytes bear HA receptors which are preferentially stimulated by exogenous high molecular weight (HMW > 5×10^5 Da) HA to produce HMW-HA, and may be inhibited by low molecular weight (LMW) HA (Ghosh *et al* 1992). The response of the synoviocytes was determined by the molecular weight and the concentration of NaHA in the local environment (Smith and Ghosh 1987). Glucosamine incorporation into HA produced by primate fibroblasts in culture is increased by exogenous NaHA (100 μg/ml) (Balazs 1985). The effects of NaHA are somewhat variable on ECM synthesis *in vitro*, with inhibition of PG synthesis by chondrocyte cultures at concentrations < 1 NaHA μg/ml, but no inhibition of muscle, skin or synovial fibroblasts up to 10 μg/ml (Ghosh *et al* 1992). Articular *in vivo* studies in dogs, rabbits and horses found decreased PG and water loss from cartilage, and lameness in response to treatment with HMW-HA and high doses of LMW-HA, the latter drug formulation having less persistent effects (Gingerich *et al* 1981; Ghosh *et al* 1992). A recent study investigated hyaluronate hexasaccharide in chondrocyte cultures (a very LMW form of HA - HA_6) and found that its presence inhibited PG synthesis by blocking the HA receptors and displacing endogenous HA (Knudson 1993). The addition of exogenous HMW-HA did not inhibit PG and ECM formation unless HA_6 was also added to the cultures.

Information on the effects of NaHA on soft connective tissues is scarce. It inhibits granulation tissue formation *in vivo*, possibly by inhibiting the chemoattraction of fibroblasts by macrophages (Rydell 1970; Rydell and Balazs 1971). In rabbits with traumatized digital tendon sheaths, treatment with HMW-HA (*mw* 1-3 x 10^6) resulted in less connective tissue reaction, smoother subcutaneous scarring and fewer and less serious adhesions (Rydell and Balazs 1971). In another study of the effect of NaHA (*mw* 1.5 x 10^6) in rabbits with partial tenotomy significant improvement in the histological appearance of the scar was not observed (Thomas *et al* 1986).

Most of the information that is available on the treatment of horses with NaHA has

been obtained in studies of joint disease. Its mechanism of action in joints is unknown; the same is true of tendons because reported studies in the horse have been clinical trials in which few or no biochemical parameters have been measured (Churchill 1985; Nixon and Gaughan 1989; Spurlock *et al* 1989a,b; Gaughan *et al* 1991; Gift *et al* 1992). There are several published reports supporting the use of NaHA for acute tendon injury, but only a few anecdotal reports of its effects with respect to return to performance activity (Churchill 1985; Nixon and Gaughan 1989; Spurlock *et al* 1989b; Gift *et al* 1992). Small studies using NaHA in an equine model of flexor tendinitis have indicated that the drug may reduce adhesion formation in acute tenosynovitis, but intratendinous healing was not improved as determined by histologic and morphometric evaluation at six weeks (Gaughan *et al* 1991; Gift *et al* 1992). However, during the six week period there was ultrasonographic evidence of a smaller lesion size, suggesting an effect on the early inflammatory response to the injury (Gift *et al* 1992). In a recent study by the same workers, sequential weekly NaHA injections using the same model did not result in significant differences in healing (Gaughan *et al* 1995). Neither mechanical nor biochemical evaluation was performed in these studies. Other reports suggest that NaHA is more beneficial in chronic than acute tendon injury, but this hypothesis has not been critically evaluated (Schmidt 1993). Despite its current use by some practitioners for equine tendinitis, NaHA has not been clearly proven to be of benefit, and further studies are required to determine its efficacy (Madison 1995; Bertone 1996).

There are several reports of the use of intrasynovial NaHA in cases of tenosynovitis of the digital sheath, with or without concurrent tendinitis of the DDFT in the horse (Barr *et al* 1995; Nixon 1996). Presumably this approach has been formulated on the basis of results in other species where adhesion formation has been reported to be reduced, and the limited results that are available for the horse (Rydell and Balazs 1971; Hagberg and Gerdin 1992; Gaughan *et al* 1991; Gift *et al* 1992; Gaughan *et al* 1995). However, a critical evaluation confirming its efficacy in the horse has not been published.

2.8.4 Polysulfated Glycosaminoglycans

Naturally occurring polysulfated glycosaminoglycans and their structural properties have been discussed. Commercially, these substances have been available as pharmaceutical agents for over 30 years and are widely used for the treatment of arthritic conditions (Dettmer 1982). They consist of preparations of naturally occurring or synthetically modified GAGs, and include Adqeuan® and Arteparon®, pentosan polysulfate (Cartrophen®) and glycosaminoglycan- peptide complex (Rumalon®) (Burkhardt and Ghosh 1987; Ghosh *et al* 1992). Recently, a number of orally administered PSGAGs (so called 'nutriceuticals') has become available, of which one, Cosequin®, is currently being investigated to determine its bioavailability and efficacy (White *et al* 1994; Hanson *et al* 1997). However, only Adequan®(PSGAG) which consists predominantly of CS purified from bovine trachea, has been investigated to a significant degree for treatment of orthopedic disease in the horse and its bioavailability confirmed for connective tissues (Andrews *et al* 1985; Burba *et al* 1993; Trotter 1996b).

Adequan® is the veterinary formulation currently in use, with intramuscular (canine and equine) and intra-articular preparations (equine) available. Arteparon® is its human and more extensively studied equivalent. The principal GAG present in PSGAG is CS, which is highly anionic due to an average of three to four sulfate esters per CS dissacharide.(Ghosh *et al* 1992). It therefore has the capacity for covalently binding with collagen, PG and noncollagen proteins, with a greater affinity for the latter two components of the connective tissue ECM (Andrews *et al* 1985). It has been determined that the drug has effects on inflammation, biosynthesis of ECM components, and ECM catabolism (Ghosh *et al* 1992).

Adequan® has been shown to have some inhibitory effects on some inflammatory reactions, including complement formation and activation (C3a and C5a), as well as reducing haemolytic complement activity (Ghosh *et al* 1992). In the horse, the inhibition of complement has been associated with a greater risk of sepsis following intra-articular medication with Adequan®. PSGAG also has an anticoagulative activity approximately one-sixth that of heparin *in vitro*, but *in vivo* studies indicate that its potency is closer to two-

thirds that of heparin (Ghosh *et al* 1992). These effects are mediated through antithrombin III. PSGAG is also able to activate prekallikreins and fibrinolysis in the presence of Hageman factor (factor VII) *in vitro*. Prostaglandin E_2 synthesis and the release of leukotriene B_4 are also inhibited by PSGAG. An antagonistic effect on interleukin-1(IL-1) has been suggested, based on findings of inhibited release of PG from cartilage, presumably due to decreased stimulation of MMPs by IL-1 (Jones and Sandstrom 1985).

Hyaluronic acid synthesis by chondrocytes and synovial cells is markedly stimulated (up to 250%) by PSGAG at 100 µg/ml, and at 200 µg/ml the average molecular weight HA was higher and had a narrower range (Greiling 1982; Burkhardt and Ghosh 1987). In synovial membrane *ex vivo* explant cultures HA synthesis was increased by DS, chondroitin-4-sulfate and chondroitin-6-sulfate, after systemic injection of rabbits. The action of PSGAG appears to result from binding to receptors on the synovial cell surface (Ghosh *et al* 1992). Increased amounts and molecular weights of PG are synthesized in a dose-dependent manner by cultured human and rabbit articular chondrocytes in response to exogenous PSGAG, but not in chick embryo or rabbit meniscus fibroblast cultures (Burkhardt and Ghosh 1987; Ghosh *et al* 1992). In osteoathritic human cartilage LP and PG synthesis (both the core protein fraction and the GAGs) was increased *in vitro*, and the mechanism responsible is thought to be direct inhibition of synthesis of the MMPs (Adam 1982).

Collagen synthesis is markedly increased by PSGAG in cartilage from chick embryos and osteoarthritic human cartilage, with the effect being more marked in the osteoarthritic cartilage. The mechanism responsible is thought to be inhibition of catabolic MMPs (Adam 1982). In another study of culture chick sternal cartilage, doses of PSGAG below 20 µg/ml did not increase collagen synthesis, but higher doses increased collagen in the medium, and collagen synthesis decreased at concentrations > than 100 µg/ml (von der Mark 1982).

The effects of Adequan® on catabolism ('chondroprotection') are widely known and are a key component of the marketing strategy used for sale of the product. It is a competitive inhibitor of neutrophil elastase and an inhibitor of cathepsin $ß_1$ (Baici and Fehr 1982; Trnavsky 1982). In addition it is a strong inhibitor of ß-glucuronidase, ß-galactosidase, ß-*N*-acetylglucosaminidase, α-glucosidase, α-mannosidase, KS glycanohydrolase,

chondroitin sulfotransferase, neutral protease, myeloperoxidase, cathepsin G, and human granulocyte elastase (Ghosh *et al* 1992). An *in vivo* lapine cartilage model showed that significant suppression of MMPs and serine proteinase activity occurred after intra-articular administration of Adequan® (Howell *et al* 1986). One *in vitro* study investigating the effect of PSGAG on equine tenocytes has been published (Dahlgren *et al* 1996). Concentrations from 25 to 200 μg/ml did not affect PG synthesis or cell viability compared to controls.

The use of glycosaminoglycans for intralesional, perilesional or systemic treatment of SDFT tendinitis is currently under investigation and although there is some support for its use, the available information is largely anecdotal (Redding *et al* 1992; Goodship *et al* 1992; Barr *et al* 1995). One injection every four days for up to seven doses has been recommended, with major reduction in pain and swelling reported by the third injection. However, one study found no significant differences between the percentage of Thoroughbred horses returning to work following a course of intratendinous or intramuscular glycosaminoglycans and those returning to work following conservative management alone (Marr *et al* 1993). Only horses with lesions less than four weeks old were treated with glycosaminoglycans, and injury recurrence rates were 50% in treated horses versus 31% in conservatively managed horses (Marr *et al* 1993).A recent clinical survey found PSGAG to be 'efficacious' in 80% of cases of SDFT tendinitis when administered intramuscularly every four days after injury for seven treatments (Dow *et al* 1996). However the cases were not standardized, the observers not blinded to the treatment, and there were no control horses. Clinical studies in human athletes with Achilles tendinitis have supported the use of intralesional glycosaminoglycans based on the clinical outcome of cases (Sundqvist *et al* 1987). Although some clinicians are advocating its use in the horse for equine tendinitis, a clear scientific rationale for its use is lacking (Barr *et al* 1995; Bertone 1996).

To date there have not been any published controlled studies evaluating orally administered GAGs in horse. In an uncontrolled, non-blinded clinical trial, orally administered PSGAG was found to be 'efficacious' for treatment of traumatic arthritis in horses (Hanson *et al* 1997). Controlled studies are underway to investigate the effect of Cosequin® on synovial and serum levels of HA and GAGs (White *et al* unpublished data).

2.8.5 Beta-aminoproprionitrile

Beta-aminoproprionitrile (ßAPN) and D-penicillinamine (D-PEN) are both agents which inhibit lysyl oxidase (lathyrogens), thus interfering with cross-linking and normal collagen maturation. ßAPN is the most widely studied and used of the two, and has recently entered the literature as a proposed treatment for equine SDFT tendinitis (Reef *et al* 1996). Although it acts by specifically and irreversibly inhibiting lysyl oxidase, ßAPN does not interfere with cross-linking once fresh lysyl oxidase is synthesized (24 hours) and the effect of its enzyme inhibition has worn off, and does not affect collagen synthesis (Chvapil 1996; Davis 1996). D-PEN however, permanently blocks collagen cross-linking by cleaving off unreduced Schiff bases, chelating Cu^{2+} and inhibiting lysyl oxidase or by complexing aldehydes formed by oxidative deaminization of hydroxyallysine or allysine (Davis 1996).

ßAPN has been recommended for topical or local administration due to toxic side effects following systemic administration in other species (Chvapil 1996). ßAPN is slowly absorbed through the skin, with ~5% of the drug diffusing into the subcutaneous tissue within 24 hours. It is rapidly absorbed by scar tissue five times faster than by normal connective tissue (Chvapil 1996). Following infusion of ßAPN the gliding and flexion of a chicken foot model of tendon adhesion was improved and peri-articular stiffening and joint fibrosis was reduced in rabbits. The infusion of ßAPN has been advocated for the treatment of equine tendinitis. Chvapil (1996) has suggested that several requirements need to be met:

a) the drug requires frequent re-administration or continuous delivery due to the rapid re-synthesis of lysyl oxidase within 24 hours of complete inhibition by ßAPN;

b) dose response effectiveness should be established;

c) knowledge of when to administer the ßAPN for optimal effect is required.

Therefore, administration in the horse should coincide with peak lysyl oxidase acyivity, ~ three to four weeks after onset of tendon injury. Recently over 200 horses with acute injury to the SDFT were treated with intralesional ßAPN (Reef *et al* 1996). Only the sonographic findings were reported, but over a 4-month period the lesion had markedly improved in response to ßAPN. However, the recommendations of Chvapil were not followed in this

study, and there are no biochemical or biomechanical data to confirm its efficacy. D-PEN has not been investigated in the horse to date, but is maintained by some workers to be potentially more effective than ßAPN (Chvapil 1996).

2.8.6 Other Drugs

Orgotein (Palosein®) has been used in the past for the treatment of equine soft tissue injuries (Linton 1976). It was recommended for its superoxide dismutase activity, its low toxicity and its chemotactic effects on polymorphonuclear leukocytes. In a case series of horses with tendinitis and other injuries, orgotein was found to be effective in resolution of acute clinical signs without side effects (Linton 1976). However the study was neither controlled nor blind, and long term follow-up of performance was not available.

Proline analogs have been used to block the synthesis of hydroxyproline, but they inhibited collagen secretion and proved too toxic for clinical use (Bora 1987; Chvapil 1996) Colchicine interferes with the secretion process, inhibiting all protein secretion and producing undesirable effects (Chvapil 1996).

CHAPTER THREE

3.0 EXPERIMENT 1: ESTABLISHMENT OF THE *IN VITRO* EXPLANT CULTURE MODEL OF THE EQUINE SDFT AND DEVELOPMENT OF ANALYTICAL PROTOCOLS

3.1 Introduction

The use of GAGs (HA and PSGAG) for intra-lesional, peri-lesional or systemic treatment of SDFT tendinitis has recently been investigated in clinical studies. Although there is some support for its use, the available information is largely anecdotal. Almost all the reported studies are of low power and are poorly controlled, and/or outcomes were determined by subjective non-blinded techniques (Schmidt 1989; Redding *et al* 1992; Goodship *et al* 1992; Marr *et al* 1993; Barr *et al* 1995). In osteoarthritis, the use of some drugs that were determined to be efficacious in clinical studies were later determined to be ineffective or harmful when controlled or laboratory oriented studies were performed (Burkhardt and Ghosh 1987). The difficulties and high risk of confounding variables involved in clinical trials has prompted many researchers to use controlled animal or tissue culture models in order to permit more rigorous biochemical and histochemical techniques in the assessment of the response to potential therapeutic agents.

The use of organ explant tissue culture for models of tendon repair has been widely supported in other species because the ECM constitutes a major portion of the tendon, and the preservation of cell-matrix interactions is considered to be important (Scott 1990). There is a substantial amount of literature suggesting that significant interactions do occur between the macromolecules of the ECM, influencing the biochemical and structural nature of matrix, as well as the migration and attachment of cells within tendon (Parry *et al* 1982; Gelberman

et al 1984; Manske *et al* 1984; Vogel *et al.* 1984; Mass and Tuel 1990; Scott 1990; Abrahamsson *et al* 1991a; Abrahamsson *et al* 1991b; Mass and Tuel 1991).

Until recently there was only one published study in which the equine SDFT tissue was cultured but neither the culture techniques nor conditions were described (Silver *et al* 1983). Tissue culture models has been accepted as a valid and useful technique for the study of the equine SDFT (Birch 1993; Dahlgren *et al* 1996; Riley *et al* 1996; Dahlgren *et al* 1997; Murphy and Nixon 1997). In each of these reports different culture techniques, sera and media were used. Only one study attempted to identify the optimal combination in the development of their model (Riley *et al* 1996). In recent publications workers have used fetal bovine serum in their cultures when evaluating drugs and cytokines (Dahlgren *et al* 1996; Murphy and Nixon 1997). There are marked differences between the composition and concentrations of molecules in fetal sera compared to serum from adult animals. Fetal serum is collected from a wide variety of fetal ages and mixed to produce a product which is infrequently characterized by the manufacturer (Barnes and Sato 1980; Jayme 1990; Abrahamsson 1991). In addition, fetal serum in high concentration has an inhibitory effect on cell growth and synthesis in culture (Jayme 1990; Abrahamsson 1991; Tornesi *et al* 1993). Adult equine serum more closely approximates the biological fluid from which nutrients diffuse into the tendons of the adult horse *in vivo* and this is important for an *in vitro* model which aims to replicate an aspect of an *in vivo* system in a controlled environment. The use of homologous serum for cultures of this type, particularly for comparative studies, has been recommended by many workers (Gelbermann *et al* 1984; Manske *et al* 1984; Minor *et al* 1986; Russell and Manske 1989). Many support using serum free media or adult mammalian serum for many tissue culture applications (Ham and McKeehan 1978; Hayashi *et al* 1978; Barnes and Sato 1980; Jayme 1990; Tornesi 1992).

Based on previous work, a modification of the equine SDFT explant model was developed in this laboratory for use in the present investigation (Riley *et al* 1996). Donor horse serum (DHS) and RPMI 1640 was utilized for the reasons outlined above, and for the improved reproducibility and reduced variability identified with this culture medium during previous studies (Riley *et al* 1996). Changes in the preparation of the equine SDFT explants

were made to standardize explant size, and establish baseline conditions for the proposed study. Although explants of the SDFT had been successfully cultured previously, it was necessary to re-establish laboratory conditions and protocols in order to minimize the risk of obtaining inconclusive or uninterpretable results (Dahlgren *et al* 1996). Thus, the following experiment was designed to establish protocols and methodology for the experiments investigating the effects of different drugs on the equine SDFT.

3.2 Objectives

1) To establish the modified *in vitro* explant culture model of the equine SDFT to be used for pharmacologic studies.
2) To validate the radiolabeling protocol proposed for use in the model.
3) To measure the variance of radioisotope incorporation rates as indices of *in vitro* biosynthesis in this model which are necessary for the planning of future studies.
4) To evaluate the suitability of bromodeoxyuridine (BrdU) labeling as a technique for quantifying the rate of cellular proliferation occurring in the equine SDFT model.
5) To determine whether or not there is segmental variation in the rates of radioisotope incorporation rates in the model.
6) To develop a reverse phase - high performance liquid chromatography (RP-HPLC) protocol for separating radiolabeled hydroxyproline and proline, to determine the relative rates of collagen and non-collagen protein synthesis..

3.3 Materials and Methods

3.3.1 Experimental Animal

One 14-month-old female Quarter horse, untrained and free from clinical evidence of tendon injury, was donated for medical reasons (Appendix I).

3.3.2 Establishment of Equine SDFT Explant Organ Cultures

The tensile, non-intrasynovial, metacarpophalangeal portions of the forelimb SDFT's were harvested under aseptic conditions from the horse within 5 minutes of death, placed in a sterile stainless steel pan containing Ringer's lactate (with 2 μg/ml amphotericin B, 100 IU/ml penicillin G sodium and 100 μg/ml gentamicin sulfate), and transferred to the tissue culture laboratory (Riley 1994). The tendons were transversely divided with a scalpel into three sections: proximal (15 to 10 cm proximal to the digital sheath; distal to the carpal sheath), middle (10 to 5 cm proximal to digital sheath) and distal (0 to 5 cm proximal to the digital sheath) thirds (ie.each portion approximately 5 cm long). Each portion was sliced transversely into 5 mm slices and, explants cut using a 4 mm diameter circular biopsy punch. The explants from each slice were individually placed in 30 ml glass Wheaton culture bottles containing 4 ml of prepared medium (RPMI 1640 with 10% DHS and 50 μg/ml ascorbate), sealed, and sterile gas (50% O_2/45%N_2/5%CO_2) injected for 10 seconds at 15 psi (Tornesi 1992; Riley 1994). Cultures were maintained on rollers (14 rpm) in a customized incubator at 36.5±0.5°C for the duration of the experiment (24 days) and the media changed (50% replacement) every 72 h unless otherwise indicated (figure 3.1).

3.3.3 Radiolabeling of Equine SDFT Explant Organ Cultures

Cultures were radiolabeled on day 24. The serum containing medium was removed from each culture and replaced with 4 ml of serum and proline free modified RPMI 1640 and 50 μg/ml ascorbate. Cultures were then radiolabeled with ^{35}S-sulfate (40 μCi/culture) and L-[2,3,4,5-^{3}H] proline (20 μCi/culture) in order to assay for sulfated glycosaminoglycan (proteoglycan) and protein (proline containing protein only) synthesis, respectively. After 24 hours, the radioactive media was removed and frozen at -20°C in stoppered vials for later analysis. The explants were then chase incubated with isotope free serum free medium for 24 hours at which time the chase medium and tissue were harvested and frozen at -20°C for later analysis.

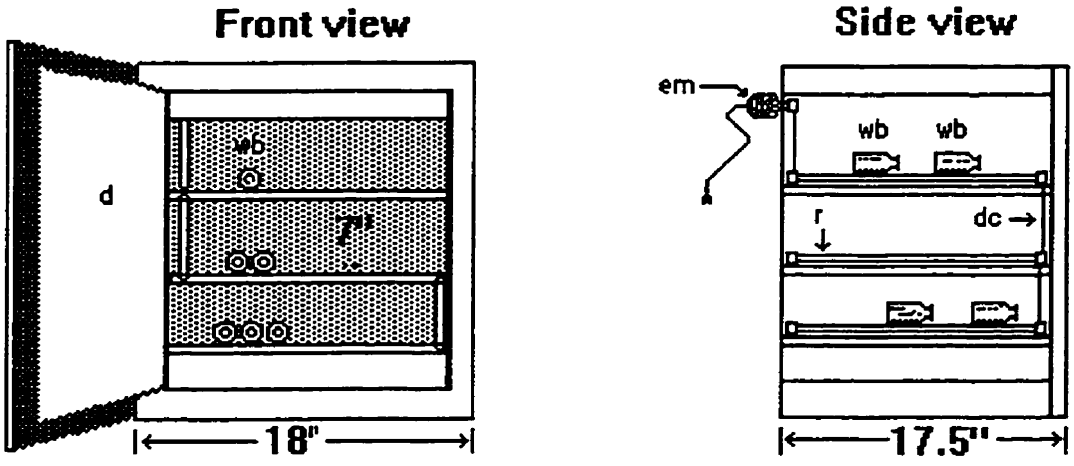

Figure 3.1. Modified roller incubator (Rousseaux 1990; Riley 1994).
d = door; dc = drive chain; em = electric motor; r = roller; wb = Wheaton bottle

3.3.4 Determination of Radioactive Isotope Incorporation Rates

Preparation and Scintillation Counting of Tendon Explants

Explants labeled with ^{35}S-sulfate and L-[2,3,4,5-^{3}H]-proline were freeze dried and weighed. Each explant was hydrolyzed in a capped 1.5 ml microfuge tube in 1 ml of 6M HCl at 110°C for 24 hours (Bailey and Light 1989). The hydrolysates were vortexed gently and a 0.5 ml aliquot transferred from each tube to a scintillation vial and evaporated to dryness by creating a gentle vortex in each vial with nitrogen gas in a hot water bath at 100°C. Samples were cleared with Solvable®, biodegradable scintillation cocktail added, and counted in a Beckman scintillation counter following the installation of quench curves for ^{35}S-sulfate and L-[2,3,4,5-^{3}H]-proline.

A microwave hydrolysis protocol was also evaluated on uncultured samples of the SDFT in 1 ml of 6M HCl placed in either in capped 1.5 ml microfuge tubes or a 7 ml scintillation vials at 800 watts for times varying from 20 seconds to 2 minutes. The vacuum centrifuge was also evaluated as a technique for drying hydrolysates.

The remaining fraction of the hydrolysates was centrifuged for 5 minutes at 3000 rpm to 1.5 ml microfuge tube with a 0.22 μm filter. The filtrates were frozen at -20°C for later derivatization with dabsyl chloride (DABS-Cl) and *O*-phtalaldehyde (OPA), and RP-HPLC.

Preparation and Scintillation Counting of Labeled Media

Biospin-6® chromatography columns (Bio-Rad) were obtained and their efficiency for removing unbound radiolabel was evaluated. This was done by adding 5 μCi of L-[2,3,4,5-^{3}H]-proline to 4 ml of culture medium. Then 100 μl aliquots were applied to each of 4 Biospin-6® columns and the eluate transferred to scintillation vials. Scintillation fluid was added to these and to 4 x 100 μl aliquots of medium which had not been passed through the columns.

Media samples harvested from the cultures were thawed, the volume of each sample measured, and the unbound radioisotope removed from 100 μl aliquots by centrifugal size exclusion chromatography using Biospin-6® columns. The eluate from each fraction was hydrolysed in 6M HCl, scintillation cocktail added, and samples were counted in a beta counter. The protein in a 1 ml aliquots of each sample of medium was precipitated in 0.15% 7-deoxycholic acid (DOCA) and 72% trichloracetic acid (TCA), centrifuged at 3300g for 30 minutes, the supernatant discarded, and the pellet air-dried and frozen for later pre-column derivatization with dabsyl chloride and *o*-phtalaldehyde (OPA), and RP-HPLC(Lin 1984; Drnevich and Vary 1993).

Calculation of Incorporation Rates of Radioactive Isotopes

Radioactivity for each culture was corrected for the specific activity (SPA) of proline in the added media and divided by the dry weight of the explant to determine the molar rates of proline incorporation, which are measures of proline-containing protein synthesized *in vitro*. ^{35}S-sulfate counts were corrected for SPA and this value divided by the dry weight of the explant to reflect the molar rate of *de novo* synthesis of sulfated glycosaminoglycans.

3.3.5 Pre-column Dabsyl Chloride Derivatization and Reverse Phase High Performance Liquid Chromatography.

Amino acid standards for OH-proline, proline, glycine, norleucine and a standard mixture of amino acids were prepared. Initially pre-column derivatization of aliquots with dabsyl chloride and OPA was used, but OPA was discontinued as the protocol was developed. Several different published solvent gradients were evaluated and modified until satisfactory separation of amino acid peaks was achieved, and development time could be minimized (Lin 1984; Jansen *et al* 1991; Drnevich and Vary 1993; Ikeda *et al* 1994). An UV absorbance detector was used to identify chromatographic peaks at 440 nm, but peak detection and resolution were poor (Moore and Stein 1948). Markedly improved peak identification was obtained with a variable wavelength UV fluorescence detector at 436 nm.

3.3.6 Identification of Proliferating Cells

Amersham's cell proliferation kit was used for monitoring cell proliferation (RPN.20)(Gratzner 1982; Matsuda 1994). The kit uses a thymidine analogue, 5-bromo-2'deoxyuridine (BrdU) which is incorporated into replicating DNA and subsequently localized using an avidin-biotin complex, specific monoclonal antibody and a peroxidase-based immunological detection system. Paraffin-impregnated 5µm sections from alcohol-fixed explants were used. The protocol was modified until satisfactory staining of nuclei with minimal non-specific staining was obtained, and qualitative evaluation was possible.

3.3.7 Statistical Analysis

All results were tabulated and their distribution and variances determined. Where the criterion for parametric testing were met, analysis of variance (ANOVA) and means comparison performed between limbs and between regions (proximal, middle and distal), with significance level set at $p < 0.05$ (Statistix 4.1, Analytical Software, FLA, USA).

3.4 Results

3.4.1 Experimental Protocols

The experimental protocols developed in this experiment were compiled into a laboratory manual which is detailed in appendix III. The chemicals, reagents and equipment used throughout the study are listed in appendix II.

Tissue Culture of Equine SDFT Explants

Explants of a uniform size were obtained using the modified protocol with the 4 mm biopsy punches. than the previously used technique, but preparation took longer (Riley *et al* 1996). Total time for set up of cultures was 7 hours. Modification of the roller incubator is required to accommodate extra cultures. Explants were successfully cultured for 24 days without antibiotics or evidence of contamination.

Radiolabeling

Both isotopes were incorporated. However, the proline incorporation was several orders of magnitude lower than sulfate incorporation.

Determination of Incorporation Rates

The microwave hydrolysis protocol, although rapid, was dangerous since some vials exploded and released HCl gas. Hydrolysis at 110^{0}C for 24 h in a water bath was preferred which, although more time consuming, was much safer. The vacuum centrifuge took several days to dry the hydrolysates, whereas the water bath and N_2 took only 6 -12 hours.

The Biospin-6® chromatography columns rapidly and efficiently removed unbound radioisotope from samples of media. Comparison of the mean count of the control (505622

dpm) with that of the chromatographically separated medium (41 dpm) indicated a 99.99% removal of isotope label from the medium ($p < 0.0001$).

The protein precipitation protocol was effective as determined by the production of a firm pellet of proteinaceous material from the culture medium.

Pre-column Derivatization and RP-HPLC

The protocol developed is outlined in table 3.1 and in more detail in appendix III. Satisfactory peak detection and resolution was obtained, with only dabsyl chloride as the fluorescent agent. The latter step simplified the derivatization protocol and facilitated the resolution of the amino acid peaks of interest. Peaks corresponding with hydroxyproline, glycine, norleucine (an internal control), and proline were separated, and the fractions corresponding to the proline and hydroxyproline were collected for scintillation counting. On chromatograms the hydroxyproline eluted as two close peaks corresponding to 4-OH-hydroxyproline and 3-OH-hydroxyproline isomers.

In order to vary temperature and decrease development time, a makeshift column heater was constructed from 2.5 mm diameter plastic tubing which was coiled carefully around the column, and the ends attached to either end of a circuit water pump and heater that was thermostatically controlled. The column and tubing were insulated with a layer of aluminum foil and foam insulation taped around the column. The thermostat of the water heater and pump were varied until the best compromise between peak resolution and separation was obtained at a constant column temperature of 32°C. The elution time for the hydrolysates was 26 minutes, followed by four to five minutes for re-equilibration of the before injection of the next sample.

Table 3.1: Isocratic gradient elution program for RP-HPLC separation of hydroxyproline and proline.

Time minutes	Flow Rate ml/min	Solvent † Concentration %		Gradient ‡
		A	B	
Initial conditions	1	100	0	
5	1	100	0	6
10	1	99	1	6
15	1	65	35	6
18	1	60	40	6
20	1	0	100	6
24	1	0	100	6
26	1	100	0	6

† Solvent A = 56% 100 mM sodium acetate, 28% methanol, 16% acetonitrile; Solvent B = 100% methanol; ‡6 = isocratic linear conditions

Identification of Proliferating Cells with Bromodeoxyuridine

Several attempts at using the BrdU assay were made. There was marked non-specific staining of non-nuclear material. This problem was overcome by using an antibody blocking step with sheep serum the protocol (see appendix III).

3.4.2 Total Proline Incorporation

Rates of proline incorporation were not significantly different among regions of the tensile portion of the SDFT (table 3.2). Incorporation rates were significantly higher for the SDFT of the right forelimb ($p < 0.05$).

Table 3.2: Mean proline incorporation per mg dry weight of tissue by region and limb.

Tendon Region	Proline Incorporation Mean ± SD pmol/mg dry wt	N
Proximal	10.16 ± 5.18	24
Middle	9.27 ± 4.81	24
Distal	10.79 ± 5.51	24
Left	7.40 ± 2.25	36
Right	12.74 ± 5.82	36
All explants	10.07 ± 5.14	72

3.4.3 Total Sulphate Incorporation

Rates of sulfate incorporation were not significantly different among regions of the tensile portion of the SDFT (table 3.3). Incorporation rates were significantly higher for the SDFT of the right forelimb ($p < 0.05$).

Table 3.3: Mean sulfate incorporation per mg dry weight of tissue by region and limb.

Tendon Region	Sulfate Incorporation Mean ± SD umol/mg dry wt	N
Proximal	18.63 ± 9.50	24
Middle	17.12 ± 8.62	24
Distal	20.58 ± 11.70	24
Left	13.56 ± 4.42	36
Right	23.99 ± 11.25	36
All explants	18.78 ± 9.98	72

3.4.4 Tendon Explant Dry Weight

There were no significant differences in tendon explant dry weight between limbs, nor among regions of the SDFT.

Table 3.4: Mean dry weight of tendon explants by limb and region.

Tendon Region	Explant Dry Weight Mean ± SD mg	N
Proximal	10.09 ± 4.03	24
Middle	11.35 ± 2.73	24
Distal	11.58 ± 3.86	24
Left	11.39 ± 3.97	36
Right	10.62 ± 3.20	36
All explants	11.00 ± 3.60	72

3.5 Discussion

The successful standardization of the explant size was confirmed by the lack of significant differences among the dry weights of different tendon regions and the relative equivalence of their variances. The disadvantage of the biopsy punch technique was the long preparation time for the cultures compared to a previously used technique, but this was reduced with experience and by changing the biopsy punch more frequently (Riley *et al* 1996). The number of explants obtained was comparable to that obtained previously and more explants may easily be obtained per horse, but further modification of the roller incubator is required to accommodate the extra cultures. On histologic examination, the reduced volume of medium from 6 to 4 ml did not appear to affect the viability of the cultures; there was little evidence of cell death or apoptosis .

The radiolabeling protocol was successful in attaining incorporation of each isotope.. However, the proline-free medium resulted in low absolute values of proline incorporation. Since the rate of proline incorporation is proportional to the proline concentration of the

medium up to a concentration optima, it is recommended that all future experiments use standard RPMI 1640 medium containing proline, and the resulting change in SPA corrected for in the calculation of results (as was done for sulfate) (Riley *et al* 1996). The actual counts reflecting sulfate incorporation were very high, so it is suggested that a lesser amount of the isotope be used in future experiments. This will reduce costs and the risk of radiation exposure.

The development of the pre-column derivatization and RP-HPLC protocols were developed by trial and error modifications of published protocols, particularly that of Drnevich and Vary (1993) (Jansen *et al* 1991; Drnevich and Vary 1993; Ikeda *et al* 1994). The explant digests were highly particulate, requiring filtration. In addition, the high concentration of native collagen prior to culture resulted in high peaks of hydroxyproline and proline, most of which are not associated with newly synthesized collagen. The protein precipitates from the culture medium was less particulate in nature and more representative of newly synthesized protein. Chromatogram peaks were also better resolved with narrower base width, facilitating fraction collection. The derivatization and RP-HPLC protocols were very sensitive to acid pH, and it was essential to maintain the pH in the region of 9 ± 0.2 units for satisfactory peak separation and resolution. The temperature of the column was determined by trial and error. Higher temperatures decreased chromatogram development time but also resulted in poorer peak separation whereas lower temperatures increased chromatogram development time.

The BrdU protocol required a blocking step with sheep serum to prevent non-specific staining. Generally cells that were stained were variable in their degree of staining and the numbers of cells that were stained was low. A large number of samples would be required for statistically meaningful quantitative comparisons using this assay. The technique is useful for determining the histologic areas in which proliferation occurs, permitting qualitative descriptions of cell proliferation. It is suggested that an alternative assay be utilized for quantitative estimates of cell proliferation, such as ^{14}C-thymidine incorporation (Baserga 1989). However, it has recently been suggested that either technique may label both proliferating cells and those undergoing DNA repair, thus providing a possible source of

measurement error (John Matyas -personal communication).

Segmental differences in protein or glycosaminoglycans synthesis were not identified, but the differences between left and right tendons were unexpected. In exercised horses differences in the mechanical and structural properties of left and right long bones have been identified, but the reason for the differences in these cultures is unclear. The differences may be associated with the medical condition for which the horse was euthanised. Future experiments will randomly assign right and left SDFT's into treatment groups.

3.6 Conclusions

1) All protocols have been satisfactorily developed but may require minor modification during future experiments (appendix III).

2) Significant regional variation in incorporation rates was not identified at the timepoint examined, but an effect related to the limb (left or right) was identified. Although this may be associated with the medical condition of the animal, random assignment to treatment groups in future experiments should take this into consideration.

3) The value of the BrdU assay appears to have limited quantitative value for this study.

4) Future experiments should use proline-containing media.

CHAPTER FOUR

4.0 EXPERIMENT 2 - PILOT STUDY A: DETERMINATION OF THE EFFECTS OF THE DURATION OF PRE-TREATMENT AND TREATMENT PERIODS ON THE METABOLIC RESPONSES OF EQUINE SDFT EXPLANTS TO POLYSULFATED GLYCOSAMINOGLYCANS, SODIUM HYALURONATE AND RECOMBINANT HUMAN IGF-I

4.1 Introduction

Tendon healing *in vitro* has many similarities with healing *in vivo*, with the exception that it proceeds at a slower rate and without systemic regulation (Gelberman *et al* 1984; Manske *et al* 1984). It is necessary to identify the *in vitro* changes in ECM synthesis that occur over time in order to determine the optimal window for drug use and evaluation in the model (Chvapil 1996). It is evident from previous *in vitro* work with the equine SDFT that differences in protein and glycosaminoglycan synthetic rates occur with time, and that variances differ depending upon the time at which endpoints are measured (Riley 1994).

Neither hyaluronate (HA or Hyonate®) nor polysulfated glycosaminoglycan (PSGAG or Adequan®) have been evaluated in cultures of the equine SDFT. Previous evaluation of the equine SDFT explant model identified significant differences in ECM synthesis among horses (Riley 1994). It is necessary to collect data in order to make some preliminary comparisons of treatment groups and information suitable for calculating the size of treatment groups in more comprehensive experiments. Studies using explant cultures of rabbit flexor tendons indicate that insulin-like growth factor-I (IGF-I) has a potent anabolic effect on protein synthesis and cell proliferation in both serum-free and supplemented media (Abrahamsson *et al* 1991a; Abrahamsson *et al* 1991b). It is possible that cultures treated

with IGF-I may be useful as a positive control in the equine SDFT model when evaluating the effects of HA and PSGAG.

The following experiment was performed to identify the mean values and distribution of data for each of the proposed treatment groups, to identify stabilization periods and duration of treatment, and to test the hypothesis that neither HA nor PSGAG will alter rates of sulfated GAGs or protein synthesis by explants of the equine SDFT.

4.2 Objectives

1) To identify the optimal pre-treatment stabilization period for the cultures prior to treatment with HA, PSGAG or rhIGF-I.
2) To identify the duration of treatment required to obtain a measurable response to treatment with HA, PSGAG or rhIGF-I.
3) To obtain data for the approximation of group sample sizes for future experiments.
4) To determine whether HA, PSGAG or rhIGF-I affect the rates of sulfated GAG or protein synthesis of explants of the equine SDFT *in vitro*.

4.3 Materials and Methods

4.3.1 Experimental Animal

One three-year-old male Quarter horse (~ 1.5 m at the shoulder) untrained and free from clinical evidence of tendon injury was purchased from the Saskatoon Auction Mart.

4.3.2 Establishment of Equine SDFT Explant Organ Cultures

The tensile portions of the forelimb SDFT's were harvested and explant cultures (196 explants) prepared as per the protocol developed in chapter three, and placed in 4 ml of prepared medium (RPMI 1640 containing proline, with 10% DHS and 50 μg/ml ascorbate),

sealed and sterile gas (50% O_2/45%N_2/5%CO_2) injected for ten seconds at 15 psi. Cultures were maintained on rollers at 36.5±0.5°C for the duration of the experiment (23 days) and the media changed (50% replacement) every 72 h unless otherwise indicated. Groups of cultures were treated according to table 4.1. For those cultures with a stabilization period of nine days, six days of treatment was proposed. However, due to the late arrival of the shipment of ^{35}S-$NaSO_4$ required for radio labeling, the treatment period was extended by one day. Doses of drugs and recombinant human (rh) IGF-I used were based on the literature, and the availability of the drugs (Abrahamsson 1991; Nethery *et al* 1992).

Table 4.1: Treatment groups

Treatment	Duration (days)			Number of Cultures
	Stabilization Period	Treatment Period	Total Culture Period*	
PSGAG††	9	7	18	12
Control	-	-	18	12
HA‡	9	7	18	12
rhIGF-I#	9	7	18	12
PSGAG†	15	6	23	16
PSGAG† & rhIGF-I#	15	6	23	16
HA‡	15	6	23	16
HA‡ & rhIGF-I#	15	6	23	16
rhIGF-I#	15	6	23	16
Control	-	-	23	20
PSGAG†	18	3	23	16
HA‡	18	3	23	16
rhIGF-I#	18	3	23	16

†Dose of PSGAG = 1000 µg/ml (Adequan®, 5 ml @ 100 mg/ml); †† dose of PSGAG = 2000 µg/ml (Adequan®, 5 ml @ 100 mg/ml, Luitpold, NY., USA); ‡dose of HA = 500 µg/ml (Hyonate®, 2.5 ml @ 10 mg/ml, Bayer Inc., ONT., Canada); # dose of rhIGF-I = 100 ng/ml (rhIGF-I 800 µl @ 250 ng/µl, Ciba Geigy, USA); *including incubation and chase periods.

4.3.3 Radiolabeling of Equine SDFT Explant Organ Cultures

Cultures were radiolabeled for 24 h at the end of each treatment period as per table 4.1. A similar protocol to experiment one was used, with the exception that the serum-free medium used for the isotopic incubations contained proline. Cultures were then radiolabeled with a reduced amount of ^{35}S-sulfate (25 μCi/culture; SPA 1497 Ci/mmol) and L-[2,3,4,5-^{3}H] proline (20 μCi/culture; SPA 114 Ci/mmol) in order to assay sulfated GAG (proteoglycan) and protein (proline containing protein only) synthesis respectively. After 24 hours, the radioactive media was removed and frozen at -20^{0}C in vials for later analysis. The explants were then chase incubated with 2 ml of isotope-free, serum-free medium for 24 hours, and then the tissue harvested and frozen at -20^{0}C for later analysis.

4.3.4 Determination of Radioactive Isotope Incorporation Rates

Preparation and Scintillation Counting of Tendon Explants

Radiolabeled explants were freeze-dried, weighed, and hydrolyzed in experiment one (Appendix III). A 0.5 ml aliquot was transferred from each tube to a scintillation vial and evaporated to dryness. Samples were cleared with Solvable®, biodegradable scintillation cocktail added, and counted to determine dpm for ^{35}S-sulfate and L-[2,3,4,5-^{3}H]-proline.

The remaining fraction of the hydrolysates was transferred to 1.5 ml microfuge tube with a 0.22 μm filter, centrifuged for 5 minutes at 3000 rpm, and the filtrates frozen at -20^{0}C for possible later analysis.

Preparation and Scintillation Counting of Labeled Media

Aliquots of 100 μl of culture medium were applied to Biospin-6® columns and the eluate transferred to scintillation vials, hydrolyzed, cocktail added, and the vials counted.

The protein in a 1 ml aliquot of each sample of medium was precipitated in 0.15%

DOCA and 72% TCA, centrifuged at 3300 g for 30 minutes, the supernatant discarded, and the pellet air dried and frozen for possible later derivatization and RP-HPLC.

Calculation of Incorporation Rates of Radioactive Isotopes

Radioactivity counts for each culture were corrected for the SPA of ^{3}H-proline and ^{35}S-sulfate, and molar rates of incorporation for the two molecules determined, reflecting measures of proline containing protein and glycosaminoglycans synthesized *in vitro*.

4.3.5 Statistical Analysis

All results were tabulated and their distribution and variances determined. Where the criterion for parametric testing were met, analysis of variance (ANOVA) and means comparisons werer performed among treatment groups. The significance level was set at $p < 0.05$, and a trend recognized at $p < 0.10$ (Statistix 4.1). For data not meeting the criterion for parametric testing, the Kruskal-Wallis non-parametric ANOVA was used.

4.4 Results

4.4.1 Total Proline Incorporation

Mean proline incorporation rates for all treatment groups are listed in table 4.2. For cultures that were stabilized for 9 days, proline incorporation rates were significantly greater in the control and rhIGF-I groups than in the HA and PSGAG groups ($p < 0.05$). In cultures that were stabilized for 18 days and treated for 3 days, there were no significant differences among treatment groups, but there was a trend for greater proline incorporation in the rhIGF-I group than the control, HA and PSGAG groups. In cultures that were stabilized for 15 days and treated for 6 days, proline incorporation rates were greater in the HA&rhIGF-I ($p < 0.05$), PSGAG ($p < 0.05$) and PSGAG&rhIGF-I ($p < 0.10$) than the control group.

Means of the two control groups were similar. The mean proline incorporation rate was significantly lower in the PSGAG group with a 9 day stabilization period than the other PSGAG groups ($p < 0.001$). There was a trend for lower mean proline incorporation rate in the HA group with a 9 day stabilization period than the other two HA groups ($p < 0.10$).

Table 4.2: Protein synthesis - Mean proline incorporation per mg dry weight of SDFT tendon by treatment group, stabilization period and duration of treatment.

Treatment	Duration (days)			Proline Incorporation Mean ± SD µmol/mg dry wt
	Stabilization Period	Treatment Period	Total Culture Period*	
PSGAG††	9	7	18	54.20 ± 18.33
Control	-	-	18	114.67 ± 26.39
HA‡	9	7	18	75.15 ± 17.27
rhIGF-I#	9	7	18	102.22 ± 17.06
PSGAG†	15	6	23	114.19 ± 19.09
PSGAG†&rhIGF-I#	15	6	23	107.88 ± 20.34
HA‡	15	6	23	90.10 ± 27.33
HA‡&rhIGF-I#	15	6	23	120.37 ± 25.10
rhIGF-I#	15	6	23	107.51 ± 13.13
Control	-	-	23	95.49 ± 14.82
PSGAG†	18	3	23	99.04 ± 23.67
HA‡	18	3	23	98.50 ± 33.39
rhIGF-I#	18	3	23	114.52 ± 17.89

†Dose of PSGAG = 1000 µg/ml; †† dose of PSGAG = 2000 µg/ml; ‡dose of HA = 500 µg/ml; # dose of rhIGF-I = 100 ng/ml; *total culture period = isotope incubation + chase incubation.

4.4.2 Total Sulfate Incorporation

Mean sulfate incorporation rates for all treatment groups are listed in table 4.3. For cultures that were stabilized for nine days, mean sulfate incorporation rates were significantly greater in the control and rhIGF-I groups than in the HA and PSGAG groups ($p < 0.01$). In cultures that were stabilized for 18 days and treated for three, there were no significant differences among treatment groups. In cultures that were stabilized for 15 days and treated for six, mean sulfate incorporation rates were greater in the HA&rhIGF-I ($p < 0.05$), PSGAG ($p < 0.10$) and PSGAG&rhIGF-I ($p < 0.10$) than the control group.

Table 4.3: Glycosaminoglycans synthesis - Mean sulfate incorporation per mg dry weight of SDFT tendon by treatment group, stabilization period and duration of treatment.

Treatment	Duration (days)			Sulfate Incorporation Mean ± SD µmol/mg dry wt
	Stabilization Period	Treatment Period	Total Culture Period*	
PSGAG††	9	7	18	87.181 ± 28.71
Control	-	-	18	172.98 ± 43.17
HA‡	9	7	18	109.67 ± 27.45
rhIGF-I#	9	7	18	150.46 ± 25.13
PSGAG†	15	6	23	176.05 ± 40.57
PSGAG† & rhIGF-I#	15	6	23	166.83 ± 45.61
HA‡	15	6	23	138.76 ± 40.40
HA‡ & rhIGF-I#	15	6	23	172.90 ± 34.75
rhIGF-I#	15	6	23	170.52 ± 23.90
Control	-	-	23	144.00 ± 21.03
PSGAG†	18	3	23	158.66 ± 35.13
HA‡	18	3	23	166.29 ± 57.24
rhIGF-I#	18	3	23	169.16 ± 35.73

†Dose of PSGAG = 1000 µg/ml; †† dose of PSGAG = 2000 µg/ml; ‡dose of HA = 500 µg/ml; # dose of rhIGF-I = 100 ng/ml; *total culture period = isotope incubation + chase incubation.

Differences between the two control groups were not significant, nor was there a trend. The mean sulfate incorporation rate was significantly lower in the PSGAG group with a nine day stabilization period than the other two PSGAG groups ($p < 0.001$). The mean sulfate incorporation rate was significantly lower in the HA group with a nine day stabilization period than the other two HA groups ($p < 0.05$).

4.4.3 Tendon Explant Dry Weight

There were no significant differences in tendon explant dry weight among treatment groups at different culture stabilization periods (9, 15 and 18 days) or following different treatment periods (three and six days in cultures maintained for 23 days) (table 4.4).

Table 4.4: Mean dry weight of SDFT tendon by treatment group, stabilization period and duration of treatment.

Treatment	Duration (days)			Explant Dry Weight Mean ± SD mg
	Stabilization Period	Treatment Period	Total Culture Period*	
PSGAG††	9	7	18	22.09 ± 4.96
Control	-	-	18	22.02 ± 5.32
HA‡	9	7	18	22.92 ± 4.32
rhIGF-I#	9	7	18	20.76 ± 3.63
PSGAG†	15	6	23	19.94 ± 5.36
PSGAG†&rhIGF-I#	15	6	23	21.29 ± 4.38
HA‡	15	6	23	20.51 ± 4.72
HA‡&rhIGF-I#	15	6	23	22.16 ± 4.27
rhIGF-I#	15	6	23	19.44 ± 4.50
Control	-	-	23	20.62 ± 4.19
PSGAG†	18	3	23	21.34 ± 5.31
HA‡	18	3	23	20.12 ± 4.70
rhIGF-I#	18	3	23	20.11 ± 2.76

†Dose of PSGAG = 1000 µg/ml; †† dose of PSGAG = 2000 µg/ml; ‡dose of HA = 500 µg/ml; # dose of rhIGF-I = 100 ng/ml; *total culture period including isotope incubation.

4.4.4 Sample Size Calculations

Sample size calculations were performed using results of cultures that were maintained for 22 days using standard statistical formulae, first using the variance of the control and then each treatment group to produce a range (table 4.5).

Table 4.5: Sample size calculations based on variances of control and treatment groups.

Treatment	Stabilization Period (days)	Treatment Period (days)	Proline Sample Size	Sulfate Sample Size
PSGAG†	15	6	24-27	17 - 26
HA‡	15	6	80-153	467 - 932
rhIGF-I#	15	6	36-85	20 - 24
PSGAG†	18	3	> 1000	33-122
HA‡	18	3	> 10,000	35 - 103
rhIGF-I#	18	3	> 300	31-41

†Dose of PSGAG = 1000 µg/ml; ‡dose of HA = 500 µg/ml; # dose of rhIGF-I = 100 ng/ml

4.5 Discussion

The choice of the culture periods chosen were based on a compromise between sampling over a four-week period (based on previous published experimental work and the lag between *in vitro* and *in vivo* healing), and maintaining adequate sample size for each group (Riley *et al* 1996). More frequent sampling of the control group may be of assistance in future for defining the growth, plateau and decline phases of cell proliferation and macromolecular synthesis for the explants. However, the system used is antibiotic free. With longer culture periods (> 4 weeks) maintaining contamination free cultures becomes increasingly difficult. In addition, with increased duration of cells in culture there is an increased risk of selecting cell line(s) associated with culture conditions to which the explants

are exposed, which are morphologically or functionally different and less representative of the original cell types present in the explant at the time of the initiation of the cultures (Freshney 1987).

The treatment times selected for comparisons were based on the practicalities of changing the medium every 3 days, and the manufactures recommendations for drug administration. PSGAG is licensed for administration every 4 days and HA every 7 days, and a course of repeated administration is recommended for both drugs. Treatment times were chosen to approximate these recommendations while not increasing the number of times cultures required medium exchange. If the medium is exchanged too frequently, the lag in cell proliferation and synthesis that occurs, may mask any responses to the drugs (Freshney 1987).

The extra treatment day for cultures with a 9 days stabilization period resulted in difficulties in making direct and relevant comparisons with the cultures stabilized for 18 days and treated for 6 days. It is possible that the apparent inhibitory effect observed in the 9 day group was associated with this difference. The dose of PSGAG used in this group also differed from the latter groups, and the effect seen may have been a toxic or inhibitory response to this concentration which was further substantiated in experiment 5 (chapter 6). The dose was reduced in the 15 and 18 day stabilization groups because of concerns about possible toxicity at such a high PSGAG concentration.

Those cultures stabilized for 15 and 18 days had the same total time in culture. However, it was only in the 15 day pre-treatment stabilization period group that significant differences were identified, suggesting that a 6 day treatment period is preferable over the 3 day treatment period or that there are significant difference between the 15 and 18 day pre-treatment culture periods. The 6 day treatment period may be preferable because there may a lag period in the response to drugs associated with a slower *in vitro* repair response than the *in vivo* response (Gelberman *et al* 1984; Manske *et al* 1984).

Although there were differences in protein and glycosaminoglycans synthesis between cultures stabilized for 9 days and those stabilized for longer periods, this did not hold true for the control groups. This latter result contrasts with previous work with the equine SDFT

in which synthetic rates for both protein and glycosaminoglycans were markedly increased after 4 weeks compared to 2 weeks of culture (Riley *et al* 1996). It is possible that the sample size in this experiment was not large enough to be representative of the control population, and sample size calculations do indicate a need for a larger number of explants per group than was used in this pilot study to determine if there is a difference.

The calculated large number of HA samples required for between the day 15 and 18 stabilization groups is high. An alternative HA dose may allow for fewer samples if there is a response to HA, indicating the need for a dose response trial.

The poor response to rhIGF-I may be due to poor bioavailabilty (either due to the IGF itself or due to the lack of suitable binding proteins in the cultures), an inappropriate concentration of the agent, or species differences in the response to IGF. An alternative source of rhIGF-I will be used in future studies.

The narrower distribution of explant dry weights for the study was indicative of improved explant standardization and homogeneity in size compared to the unmodified model (Riley 1994). This represents an improvement in the model, and enhances its suitability for the study of equine SDFT explants to different drugs and biological agents.

4.6 Conclusions

1) Rates of proline and sulfate incorporation were stable in the cultures over the 18 to 23 days total culture period.

2) An apparently inhibitory effects on proline and sulfate incorporation rates due to PSGAG at 2000 µg/ml (double the dose used for later cultures) were observed compared to controls in explants cultured for 9 days and treated for 7 days.

3) Rates of proline and sulfate incorporation were increased in the presence of PSGAG at 1000 µg/ml compared to controls in explants cultured for 15 days and treated for 6 days.

4) The results for rhIGF-I at 100 ng/ml did not support its use as a positive control.

5) The narrower distribution of dry weights for the explants indicates improved standardization of explant size than the previously used technique (Riley 1994).

CHAPTER FIVE

5.0 EXPERIMENT 3 - PILOT STUDY B: DETERMINATION OF THE EFFECTS OF THE DURATION OF PRE-TREATMENT AND TREATMENT PERIODS ON THE METABOLIC RESPONSES OF EQUINE SDFT EXPLANTS TO POLYSULFATED GLYCOSAMINOGLYCANS AND RECOMBINANT HUMAN IGF-I

5.1 Introduction

In the previous experiment (pilot study A) it was determined that the response to treatment differed depending upon the duration of pre-treatment and/or treatment periods. Experimental design was sub-optimal, resulting in difficulties in making meaningful and drawing conclusions.

Sample size calculations indicated that PSGAG (at 1000 μg/ml) and rhIGF-I, but not HA, are suitable for evaluation at the concentrations tested. A dose response study was necessary before proceeding further with other HA studies. Therefore in this experiment HA was not evaluated. Dose response studies for both HA and PSGAG were performed later.

The results for rhIGF-I (Ciba-Geigy) did not support its use as a positive control. The source used had not been tested for its suitability for *in vitro* studies. Therefore an alternative source that had tested for its suitability for *in vitro* studies was used (Upstate Biotechnology Inc., NY., USA).

This experiment will test the hypothesis that differences in the pre-treatment stabilization culture period will not significantly affect rates of sulfated GAG and protein synthesis in either the control or treatment groups.

5.2 Objectives

1) To compare the effects of different pre-treatment culture stabilization periods on the protein and sulfated GAG synthetic rates of equine SDFT explant cultures.
2) To determine the effects of PSGAG and rhIGF-I and on the protein and sulfated GAG synthetic rates of equine SDFT explant cultures at each different culture period (stabilization period plus treatment period).
3) To obtain further data for the approximation of group sample sizes for future experiments.

5.3 Materials and Methods

5.3.1 Experimental Animal

One two-year-old male Quarter horse cross (~ 1.5 m at the shoulder), untrained and free from clinical evidence of tendon injury, was obtained from the Saskatoon Auction Mart.

5.3.2 Establishment of Equine SDFT Explant Organ Cultures

The tensile portions of the forelimb SDFT's were harvested and explant cultures prepared as per the protocol (appendix III). Cultures were maintained on rollers at 36.5 ± 0.5^{0}C for the duration of the experiment (29 days) and the media changed (50% replacement) every 72 h unless otherwise indicated. A total of 221 cultures were obtained. The pre-treatment culture period for different groups of cultures are outlined in table 5.1. All cultures were treated (or not treated in the control group) for six days before radiolabeling. The concentration of PSGAG used was 1000 μg/ml. The concentration of rhIGF-I used was based on the manufacturers recommendations (100 ng/ml).

Table 5.1: Number of explants in each treatment group.

Treatment	Pre-treatment Stabilization Period (days)		
	9††	15†‡	21‡‡
PSGAG†	18*	18	20
PSGAG† & rhIGF-I‡	18	18	19
rhIGF-I‡	18	18	18
Control	18	18	20

††Radiolabeled on day 15; †‡radiolabeled on day 21; ‡‡radiolabeled on day 27; †Dose of PSGAG = 1000 µg/ml (Adequan®, 5 ml @ 100 mg/ml, Luitpold Pharmaceuticals, NY., USA); ‡dose of rhIGF-I = 100 ng/ml (Upstate Biotechnology, 250 ng/µl, NY., USA); *one explant lost during freeze drying.

5.3.3 Radiolabeling of Equine SDFT Explant Organ Cultures

Cultures were radiolabeled for 24 h at the end of each treatment period as per table 5.1 (pre-treatment stabilization period plus treatment period). Cultures were radiolabeled and chase incubated as per the previously outlined protocol [^{35}S-sulfate (25 µCi/culture; SPA 1497 Ci/mmol) and L-[2,3,4,5-^{3}H] proline (20 µCi/culture; 114 Ci/mmol)](appendix III).

5.3.4 Determination of Radioactive Isotope Incorporation Rates

Preparation and Scintillation Counting of Tendon Explants

Explants were freeze dried, weighed, hydrolyzed and radioactivity determined as per the protocol developed in experiment one (Appendix III).

Preparation and Scintillation Counting of Labeled Media

Aliquots of 100 µl of culture medium were applied to Biospin-6® columns and the eluate transferred to scintillation vials, hydrolyzed, cocktail added, and the vials counted.

Calculation of Incorporation Rates of Radioactive Isotopes

Radioactivity counts for each culture were corrected for the specific activity of ^{3}H-proline and ^{35}S-sulfate, and molar rates of incorporation for the two molecules determined, reflecting measures of proline containing protein and sulfated GAGs synthesized *in vitro*.

5.3.5 Statistical Analysis

All results were tabulated and their distribution and variances determined. Where the criterion for parametric testing were met, analysis of variance (ANOVA) and a means comparison performed among treatment groups. The significance level was set at $p < 0.05$, with a trend recognized at $p < 0.10$ (Statistix 4.1). For data not meeting the criteria for parametric testing, the Kruskal-Wallis non-parametric ANOVA was used.

5.4 Results

5.4.1 Total Proline Incorporation

Mean proline incorporation rates for all groups are listed in table 5.2 (figure 5.1). For cultures that were stabilized for either nine days or 15 days prior to treatment, mean proline incorporation rates were not significantly different among drug treatment groups ($p > 0.10$). In cultures that were stabilized for 21 days, mean proline incorporation rates were not significantly different among groups ($p > 0.10$). Differences among the three control groups (pre-treatment culture periods 9, 15 and 21) were significant between the 9 and 21 day controls only ($p < 0.05$). The day 15 control group did not differ significantly from either of the two other groups, nor was there a trend.

The mean proline incorporation rate was significantly lower in the PSGAG group with a 9 day stabilization period than the 21 day group ($p < 0.02$). The 15 day stabilization PSGAG group did not differ significantly from the two other PSGAG groups.

Table 5.2: Protein synthesis - Mean proline incorporation per mg dry weight of equine SDFT by treatment group and pre-treatment stabilization period.

Treatment	Proline Incorporation Rates Mean ± SD µmol/mg dry wt		
	Pre-treatment Stabilization Period (Days)		
	9††	15‡‡	21‡‡
PSGAG†	97.70 ± 16.87	106.47 ± 34.99	177.91 ± 131.09
PSGAG† & rhIGF-I‡	106.52 ± 21.89	106.53 ± 33.59	147.27 ± 57.62
rhIGF-I‡	103.88 ± 27.18	117.37 ± 38.33	141.76 ± 35.68
Control	90.90 ± 21.00	107.67 ± 33.77	128.03 ± 45.90

††Radiolabeled on day 15; ‡‡radiolabeled on day 21; ‡‡radiolabeled on day 27; †Dose of PSGAG = 1000 µg/ml; ‡dose of rhIGF-I = 100 ng/ml.

The mean proline incorporation rate was significantly higher in the PSGAG & rhIGF-I group with a 21 day stabilization period than the other two PSGAG & rhIGF-I groups ($p < 0.05$). The 15 day stabilization PSGAG& rhIGF-I group did not differ significantly from the 9 day stabilization group, nor was there a trend.

The mean proline incorporation rate was significantly higher in the rhIGF-I group with a 21 day stabilization period than the other two rhIGF-I groups ($p < 0.05$). The 15 day stabilization rhIGF-I group did not differ significantly from the 9 day stabilization group, nor was there a trend.

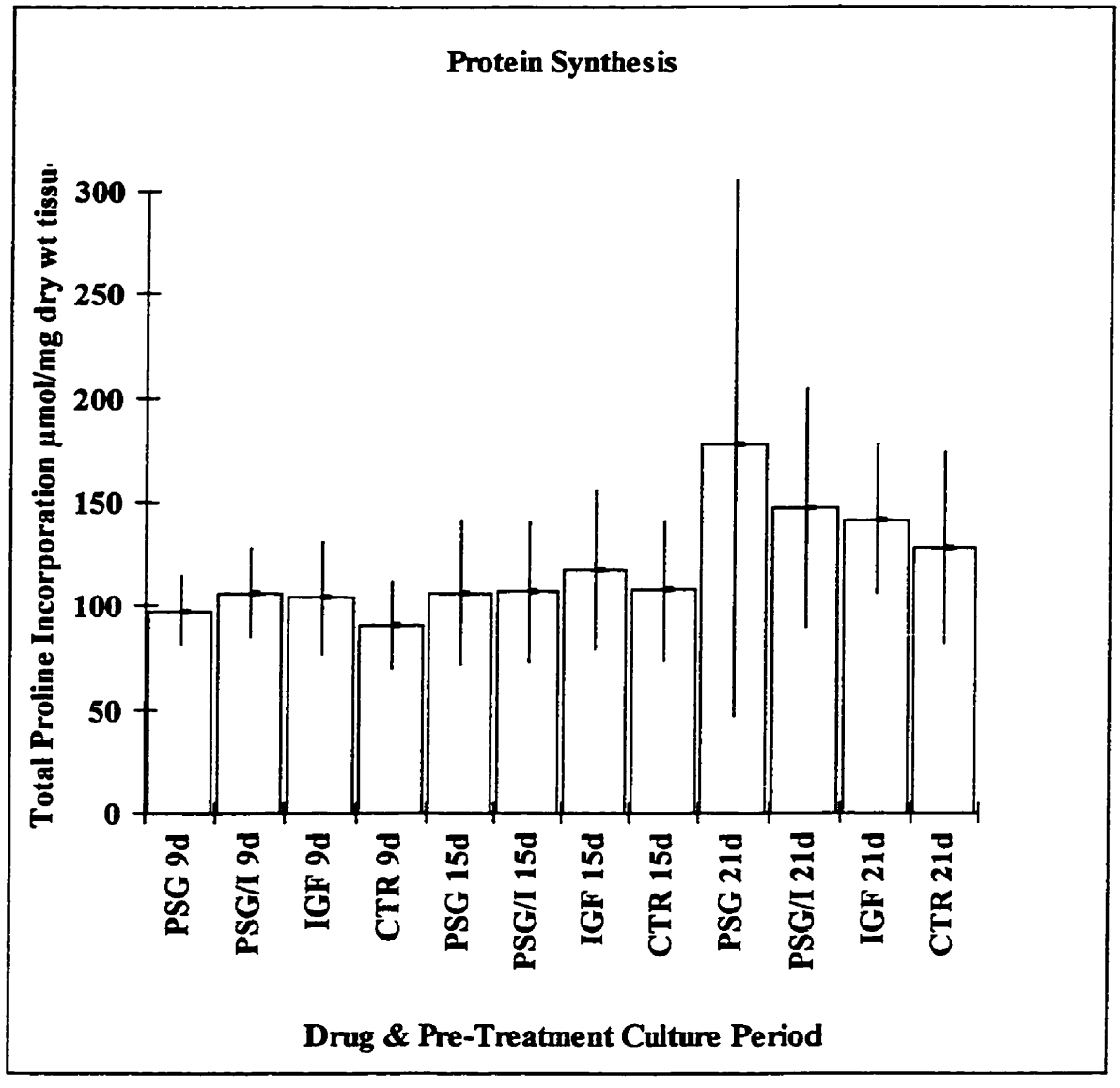

Figure 5.1: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by treatment group and duration of pre-treatment culture period (mean ± sd µmol/mg dry weight of tendon). PSG = polysulfated glycosaminoglycans; PSG/I = polysulfated glycosaminoglycans & IGF-I; IGF = IGF-I; CTR = control; d = days.

5.4.2 Total Sulfate Incorporation

Mean sulfate incorporation rates for all treatment groups are listed in table 5.3 (figure 5.2). For cultures that were stabilized for nine days prior to the six days of treatment, mean sulfate incorporation rates were not significantly different among drug treatment groups ($p > 0.10$). In cultures that were stabilized for 15 days or 21 days there were no significant differences in mean sulfate incorporation rates among treatment groups ($p > 0.10$).

Among the three control groups (pre-treatment culture periods 9, 15 and 21) the mean sulfate incorporation rate was significantly lower in the nine day pre-treatment culture group than the 15 and 21 day controls ($p < 0.05$). The day 15 control group did not differ significantly from the day 21 group, nor was there a trend.

The mean sulfate incorporation rate was significantly lower in the PSGAG group with a nine day stabilization period than the 21 day PSGAG group ($p < 0.05$). The 15 day stabilization PSGAG group did not differ significantly from either of the other PSGAG groups, nor was there a trend.

Table 5.3: Glycosaminoglycans synthesis - Mean sulfate incorporation per mg dry weight of equine SDFT by treatment group and pre-treatment stabilization period.

Treatment	Sulfate Incorporation Rates Mean ± SD µmol/mg dry wt		
	Pre-treatment Stabilization Period (Days)		
	9††	15†‡	21‡‡
PSGAG†	102.33 ± 22.61	121.29 ± 48.37	194.97 ± 148.31
PSGAG†&rhIGF-I‡	102.12 ± 22.05	122.87 ± 45.98	151.81 ± 76.24
rhIGF-I‡	108.70 ± 30.49	143.92 ± 70.62	132.94 ± 37.68
Control	93.30 ± 35.52	134.31 ± 41.83	134.63 ± 63.91

††Radiolabeled on day 15; †‡radiolabeled on day 21; ‡‡radiolabeled on day 27; †Dose of PSGAG = 1000 µg/ml; ‡dose of rhIGF-I = 100 ng/ml.

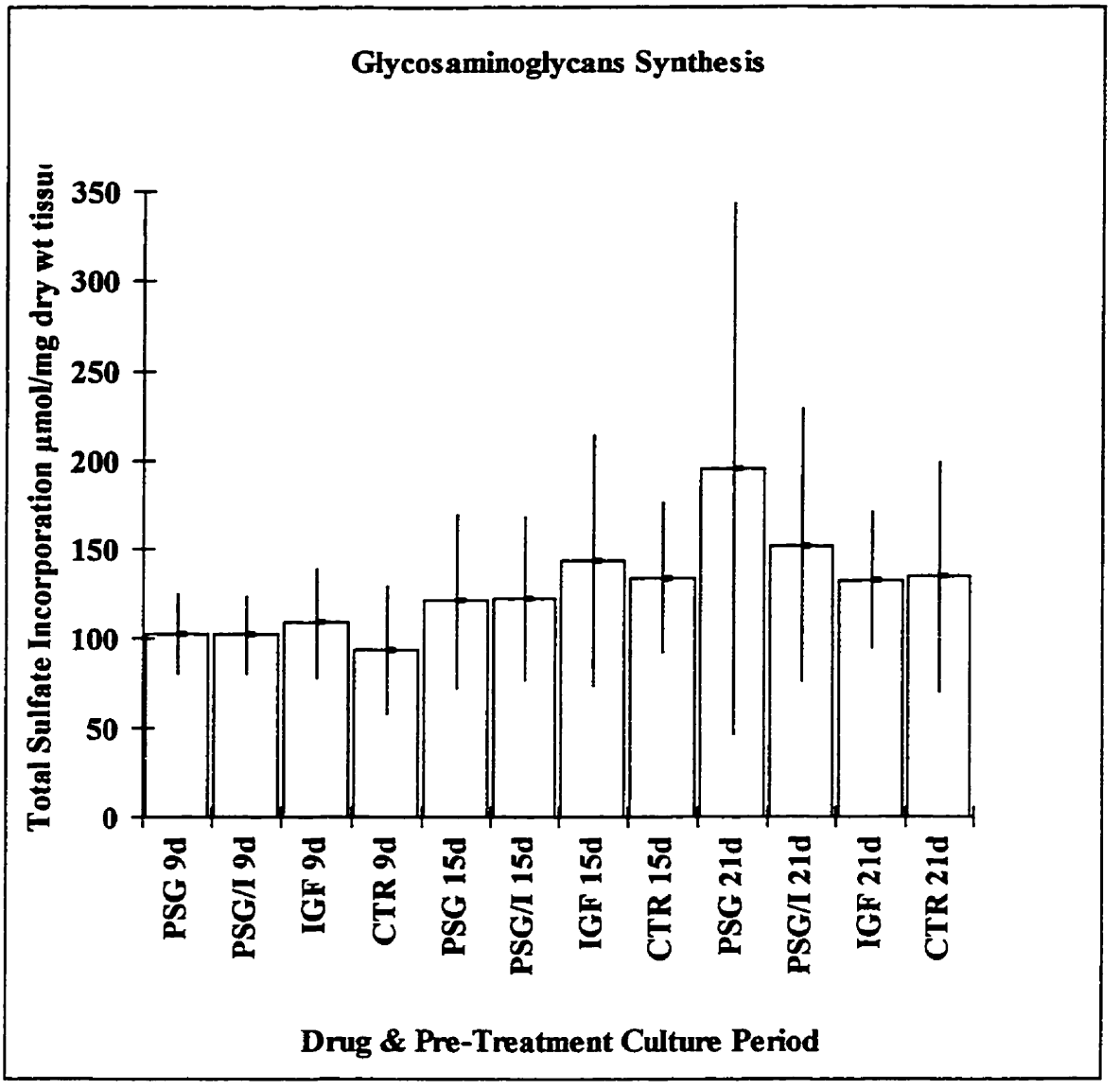

Figure 5.2: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by treatment group and duration of pre-treatment culture period (mean ± sd µmol/mg dry weight of tendon). PSG = polysulfated glycosaminoglycans; PSG/I = polysulfated glycosaminoglycans & IGF-I; IGF = IGF-I; CTR = control; d = days.

The mean sulfate incorporation rate was significantly lower in the PSGAG&rhIGF-I group with a nine day stabilization period than the 21 day PSGAG&rhIGF-I group ($p < 0.05$). The 15 day stabilization PSGAG&rhIGF-I group did not differ significantly from either of the two other PSGAG&rhIGF-I groups, nor was there a trend. There were no differences among the three rhIGF-I groups in mean sulfate incorporation rates ($p > 0.10$).

5.4.3 Tendon Explant Dry Weight

There were no significant differences in mean equine SDFT explant dry weights among drug treatment groups for explants stabilized for nine, 15, or 21 days (table 5.4; figure 5.3). However, the mean dry weight of all explants stabilized for nine days was significantly greater than the other two stabilization periods ($p < 0.05$), and the mean dry weight of all explants stabilized for 15 days was significantly greater than the mean dry weight of cultures stabilized for 21 days ($p < 0.05$).

Table 5.4: Mean dry weight of equine SDFT explants by treatment group and pre-treatment stabilization period.

Treatment	Explant Dry Weight Mean ± SD mg		
	Pre-treatment Stabilization Period (Days)		
	9††	15†‡	21‡‡
PSGAG†	18.38 ± 3.79	16.73 ± 3.82	9.54 ± 4.38
PSGAG†&rhIGF-I‡	19.31 ± 3.97	18.04 ± 7.54	11.52 ± 4.43
rhIGF-I‡	18.59 ± 3.94	15.45 ± 5.73	13.19 ± 5.08
Control	17.87 ± 6.59	16.75 ± 5.73	13.08 ± 5.64

††Radiolabeled on day 15; †‡radiolabeled on day 21; ‡‡radiolabeled on day 27; †Dose of PSGAG = 1000 µg/ml; ‡dose of rhIGF-I = 100 ng/ml.

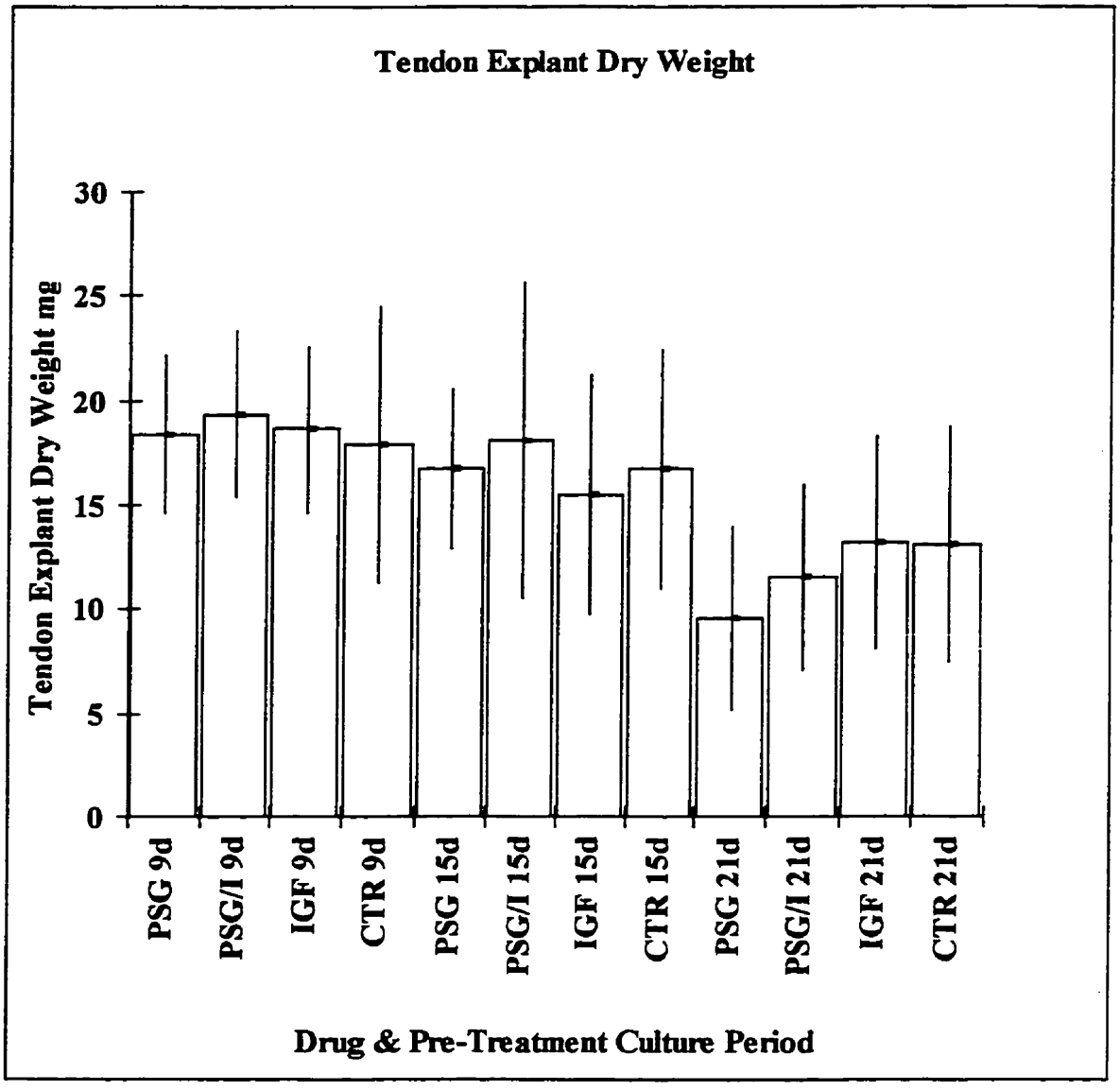

Figure 5.3: Tendon Explant Dry Weight - Mean equine SDFT explant dry weight by treatment group and duration of pre-treatment culture period (mean ± sd mg dry weight of tendon). PSG = polysulfated glycosaminoglycans; PSG/I = polysulfated glycosaminoglycans & IGF-I; IGF = IGF-I; CTR = control; d = days.

5.4.4 Sample Size Calculations

Sample size calculations were performed for results of the controls and PSGAG treatment groups to determine how large groups were needed to detect a significant effect.. Calculations were determined using standard statistical formulae, using the variance of the control and then each treatment group to produce a range (table 5.5).

Table 5.5: Sample size calculations based on variances of control and treatment groups.

Treatment	Stabilization Period (days)	Treatment Period (days)	Proline Sample Size	Sulfate Sample Size
PSGAG†	9	6	97 -150	93-236
Control	9	6	97 -150	93-236
PSGAG†	15	6	160 - 13000	160 - 13000
Control	15	6	160 - 13000	160 - 13000
PSGAG†	21	6	36-110	27-566
Control	21	6	36-110	27-566

†Dose of PSGAG = 1000 µg/ml.

5.5 Discussion

The significance of the results from this experiment are questionable given the findings of a decline in explant mass during the experiment, and the fact that data was normalized on the basis of dry weight. Normalization of data based on the amount of DNA per explant may have improved the accuracy of comparisons, but extracting DNA from equine tendon tissue is difficult, and results in low yields because of the resistance of the tissue to disaggregation and its low cellular density (Birch 1993). The decline in explant dry weights contrasts with the results of experiments one and two, and with previous work with this model in which significant differences among explant dry weights over time were not

detected (Riley 1994). There are sseveral possible reasons for this decline: 1) poor homogeneity in explant preparation during the establishment of cultures; 2) cumulative toxic effects from the medium or serum; 3) contamination of the cultures with mycoplasma; 4) progressive catabolism of the ECM over time.

The explant preparation procedures were identical to those used for the previous two experiments, and explants appeared to be of similar size. This possibility would not explain the reduction in explant size across all treatment groups with successive pre-treatment culture periods. The same batch and concentration of serum, and the same batch of RPMI 1640 medium was used in this experiment as the previous two experiments. Since neither turbidity nor marked acidity was noted in the cultures, bacterial infection was not indicated in the cultures. Therefore the most likely explanation for the reduction in explant size is infection with mycoplasma organisms (Freshney 1987). This possibility is further supported by the high variances of data within each treatment group, particularly in the cultures stabilized for 21 days prior to treatment. It is therefore recommended for all future experiments that representative sample be submitted for mycoplasma isolation.

It appears that a pre-treatment culture stabilization period greater than 15 days is preferable for future studies based on the results. However the reduction in explant size (possibly due to mycoplasma) may have artificially increased the values obtained for those cultures pre-incubated for 21 days.

Within each pre-treatment culture period, rhIGF-I failed to increase protein or glycosaminoglycan synthesis at the concentration tested. Although the dose used was high, based on previous literature, the equine SDFT explants may be less responsive than other tissues (Abrahamsson 1991). Therefore a dose response study for rhIGF was recommended. The response of explants to PSGAG observed in experiment two was not detected in this experiment. This may be due to an inappropriate dose, or a reflection of the possible effect of mycoplasma infection of the cultures. A dose response study is necessary for PSGAG.

5.6 Conclusions

1) Rates of synthesis are optimized by a pre-treatment culture period greater than 15 days.
2) Neither PSGAG nor rhIGF-I altered the rates of synthesis of protein or sulfated GAGs compared to controls within each pre-treatment culture period group.
3) Dose response studies for PSGAG and rhIGF-I are required.
4) The interpretation of results from this experiment are confounded by the decline in explant size during the experiment, and a high probability of mycoplasma infection of the cultures.
5) A representative sample from future experiments should be tested for mycoplasma.

CHAPTER SIX

6.0 EXPERIMENT 4 : EVALUATION OF THE EFFECT OF DOSE ON THE METABOLIC RESPONSES OF EQUINE SDFT EXPLANTS TO POLYSULFATED GLYCOSAMINOGLYCANS AND RECOMBINANT HUMAN IGF-I

6.1 Introduction

The results from the previous three experiments suggest that a pre-treatment culture period of 15 to 21 days and a treatment period of six days as suitable initial conditions for the study of the response of the equine SDFT explants to different pharmacologic and biologic agents.

In the previous experiments it was determined that the response to treatment with PSGAG differed depending upon the pre-treatment stabilization period. Although a trend was observed in experiment two, significant differences in synthesis were not observed following treatment with PSGAG at a dose of 1000 μg/ml when compared to controls. Statistical power calculations using the results of experiments two and three confirmed the need for larger treatment groups for comparisons. Additionally, the effect of different concentrations may be an important determinant of the degree of response, if any, of the SDFT explant cultures to PSGAG.

The addition of rhIGF-I (at doses ranging from ten to 1000 ng/ml) to explant cultures of the flexor tendons has resulted in the stimulation of both sulfated GAG and collagen synthesis in a dose dependent manner in the rabbit, with maximal stimulation at 250 ng/ml (Abrahamsson *et al* 1991a&b). Recently an equine study similar to that performed in the rabbit reported an increase in collagen but not sulfated GAG synthesis in response to rhIGF-I at 100 and 250 ng/ml (but not 500 ng/ml) (Murphy and Nixon 1997). The results of this

study are difficult to interpret because the cultures were treated with rhIGF-I in the presence of 5% fetal bovine serum (FBS) which has high concentrations of growth factors including IGF-I, is not species specific, and varies widely from batch to batch in its composition (Jayme 1990; Riley 1994). Experiments two and three used rhIGF-I at 100 ng/ml in the presence of 10% DHS (same batch), but in both cases rhIGF-I alone did not increase synthetic rates compared to controls at the concentration tested. Previous studies have suggested that the response to rhIGF-I is dependent upon its concentration in the medium. It is necessary to determine if such a relationship exists when the equine SDFT explants in the current study are treated with rhIGF-I.

This aims of this experiment are to test the hypotheses that 1) The addition of PSGAG at different concentrations to explant cultures of the equine SDFT will not significantly affect rates of sulfated GAG and protein synthesis compared to a control group, and 2) The addition of rhIGF-I at different concentrations to explants of the equine SDFT will not significantly affect rates of sulfated GAG and protein synthesis compared to a control group of cultures.

6.2 Objectives

1) To determine the effects of different concentrations of PSGAG in the culture medium on the rates of protein and sulfated GAG synthesis by explant cultures of the equine SDFT.
2) To determine the effects of different concentrations of rhIGF-I in the culture medium on the rates of protein and sulfated GAG synthesis by explant cultures of the equine SDFT.

6.3 Materials and Methods

6.3.1 Experimental Animal

A three and one half-year-old female Thoroughbred cross (~ 1.5 m at the shoulder), untrained and free from clinical evidence of tendon injury, was purchased from the Saskatoon Auction Mart.

6.3.2 Establishment of Equine SDFT Explant Organ Cultures

The tensile portions of the forelimb SDFT's were harvested and explant cultures prepared using the previously developed protocol (appendix III). Cultures were maintained on rollers at 36.5±0.5°C for the duration of the experiment (27 days) and the media (RPMI 1640 containing proline, with 10% DHS and 100 µg/ml ascorbate) changed every 72 h unless otherwise indicated (Russell and Manske 1991). After a pre-treatment period of 18 days, cultures were treated with different doses of either PSGAG (Adequan®, Luitpold Pharmaceuticals, NY., USA) or rhIGF-I (Upstate Biotechnology Inc., NY., USA) for seven days prior to radiolabeling (table 6.1)(Nethery *et al* 1992). A total of 212 cultures were obtained; six were randomly selected and tested for mycoplasma at day 18.

Table 6.1: Treatment groups for PSGAG and rhIGF-I dose response trials.

Treatment	Concentration µg/ml	Number of Explants	Treatment	Concentration ng/ml	Number of Explants
PSGAG†	100	30	Control	0	30
PSGAG†	500	30	rhIGF-I‡	50	8
PSGAG†	1000	30	rhIGF-I‡	100	8
PSGAG†	2000	30	rhIGF-I‡	250	8
PSGAG†	5000	30	rhIGF-I‡	500	8

†Adequan® 100 mg/ml, Luitpold Pharmaceuticals, NY., USA; ‡rhIGF-I diluted to 160 ng/µl, Upstate Biotechnology Inc., NY., USA.

6.3.3 Radiolabeling of Equine SDFT Explant Organ Cultures

Cultures were radiolabeled [^{35}S-sulfate (20 µCi/culture; SPA 1497 Ci/mmol) and L-[2,3,4,5-^{3}H] proline (20 µCi/culture; SPA 114 Ci/mmol)] for 24 h at the end of the treatment period (day 25) and chase incubated for 12 hours using the established protocol with 2 ml of serum free medium. It was planned that cultures would be labeled with methyl-C^{14}-thymidine (1 µCi/culture) as an index of cell proliferation, but due to failure of the shipment to arrive, this was not possible.

6.3.4 Determination of Radioactive Isotope Incorporation Rates

Determination of Incorporation Rates of Radioactive Isotopes by Tendon Explants

The preparation and scintillation counting of the tendon explants and of the labeled media, as well as the calculation of incorporation rates were performed as described in Appendix III.

Pre-column Derivatization and RP-HPLC

Aliquots of culture medium were precipitated in 0.15% DOCA and 72%TCA, incubated for 10 mins at room temperature, centrifuged for 30 minutes at 3300g and the supernatant decanted. Each protein pellet was digested in 6M HCl for 24 hours at 110^{0}C and then the pH adjusted to 9.0. Aliquots of 40 or 45 µl were derivatized with DABS-Cl and then Hyp and Pro separated by RP-HPLC. Fractions corresponding to the Hyp and Pro peaks were collected, scintillation cocktail added and the fractions counted on a beta counter. The ratio of the counts and a formula accounting for the relative concentrations of Hyp and Pro in collagen were used to determine molar rates per mg dry weight of proline incorporation into collagen and noncollagen protein precipitated from the medium (ie. soluble collagen) .

6.3.5 Statistical Analysis

All results were tabulated and their distribution and variances determined. Variances were not found to be equivalent, therefore the Kruskal-Wallis non-parametric ANOVA was used and a means comparison performed among treatment groups, with a significance level set at $p < 0.05$, with a trend recognized at $p < 0.10$.

6.4 Results

The results of mycoplasma cultures were negative. Explants < 6 mg dry weight (mean 3.1 mg; n = 13) were excluded from the analysis, on the basis that results for these were subject to high measurement error due to low dry weight. Their removal ensured that dry weights were comparable among all groups.

6.4.1 PSGAG Treated Cultures - Total Protein Synthesis

Mean total proline incorporation rates were significantly greater for the 5000 µg/ml ($p < 0.01$), 2000 µg/ml ($p < 0.05$), 1000 µg/ml ($p < 0.01$) and 500 µg/ml ($p < 0.05$) PSGAG treatment groups than the control group (table 6.2). The mean total proline incorporation rates were significantly greater for the 5000 µg/ml and 1000 µg/ml than the 100 µg/ml PSGAG group ($p < 0.05$), giving statistical support for the apparent dose-related response observed in figure 6.1.

Table 6.2: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of PSGAG in the medium.

PSGAG Concentration in Medium (µg/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*		
0	72.06 ± 26.22	A		
100	79.69 ± 12.67	A	B	
500	95.84 ± 40.77		B	C
1000	118.07 ± 47.89			C
2000	93.33 ± 49.55		B	C
5000	96.66 ± 28.20			C

* Common letters denote that groups were not significantly different from each other

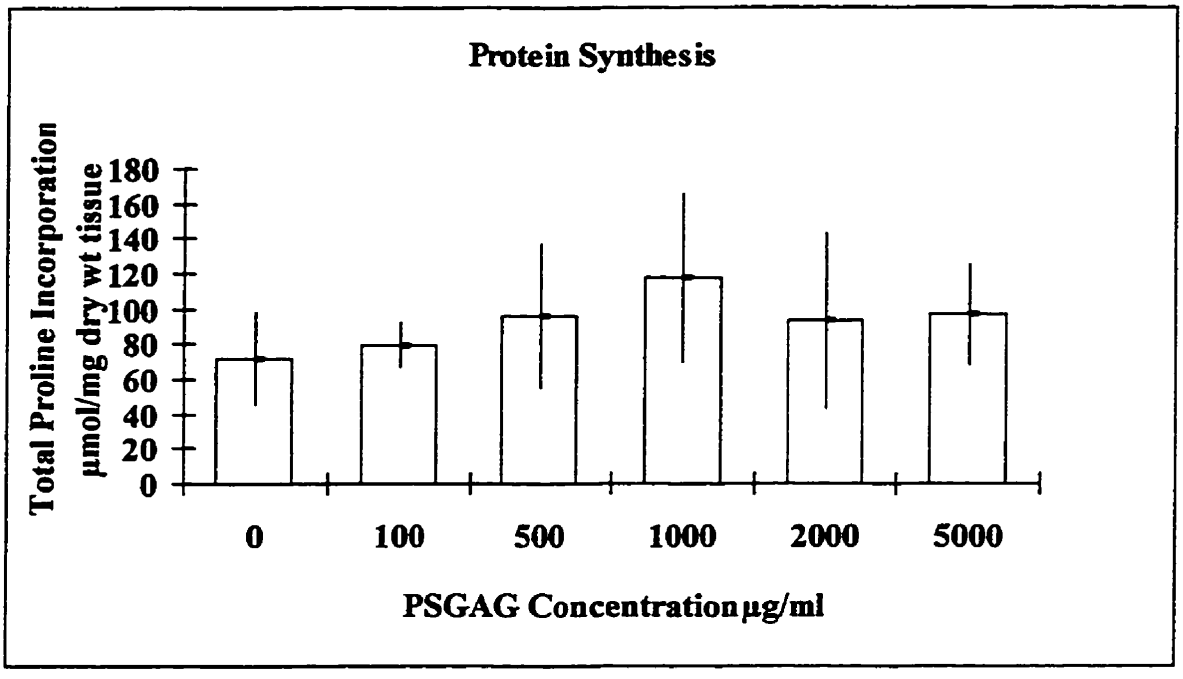

Figure 6.1: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of PSGAG in the culture medium (mean + sd µmol/mg dry weight of tendon).

6.4.2 PSGAG Treated Cultures - Collagen Synthesis

Mean proline incorporation rates into collagen were significantly greater for the 5000 µg/ml ($p < 0.01$), 2000 µg/ml ($p < 0.05$), 1000 µg/ml ($p < 0.01$) and 500 µg/ml ($p < 0.01$) PSGAG treatment groups than the control group (table 6.3). The rate of collagen synthesis was greater in the 5000 µg/ml, 2000 µg/ml, 1000 µg/ml than the 100 µg/ml PSGAG treatment group ($p < 0.05$), lending some statistical support to the apparent dose response observed in figure 6.2.

Table 6.3: Collagen synthesis - Mean proline incorporation rate into collagen† per mg dry weight of equine SDFT by concentration of PSGAG in the medium.

PSGAG Concentration in Medium (µg/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*		
0	7.57 ± 7.35	A		
100	10.77 ± 6.67	A	B	
500	19.76 ± 13.47		B	C
1000	35.41 ± 25.55			C
2000	35.02 ± 17.90			C
5000	26.30 ± 17.99			C

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

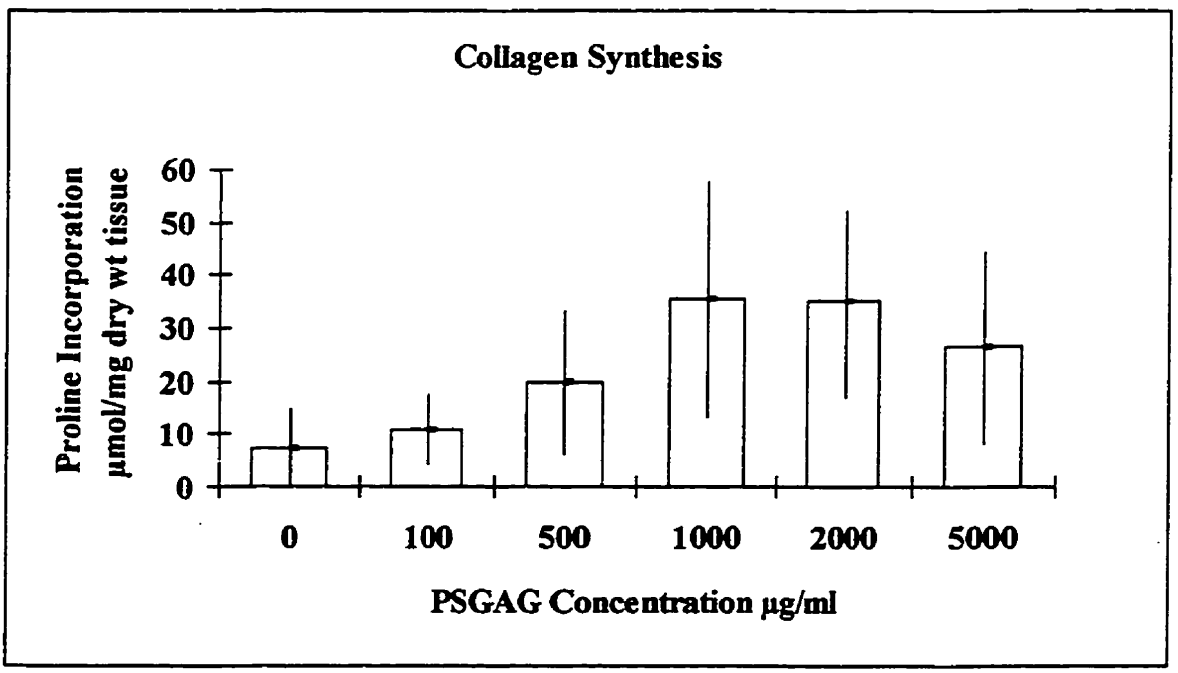

Figure 6.2: Collagen synthesis - Mean rate of proline incorporation into collagen (in the medium) per mg dry weight of equine SDFT by concentration of PSGAG in the culture medium (mean + sd µmol/mg dry weight of tendon).

6.4.3 PSGAG Treated Cultures - Noncollagen Protein Synthesis

Mean proline incorporation rates into noncollagen protein were significantly greater for the all PSGAG treatment groups than the control group ($p < 0.05$ for the 2000 µg/ml group; $p < 0.01$ for all other PSGAG groups) (table 6.4). An apparent dose response illustrated in figure 6.3 could not be supported statistically.

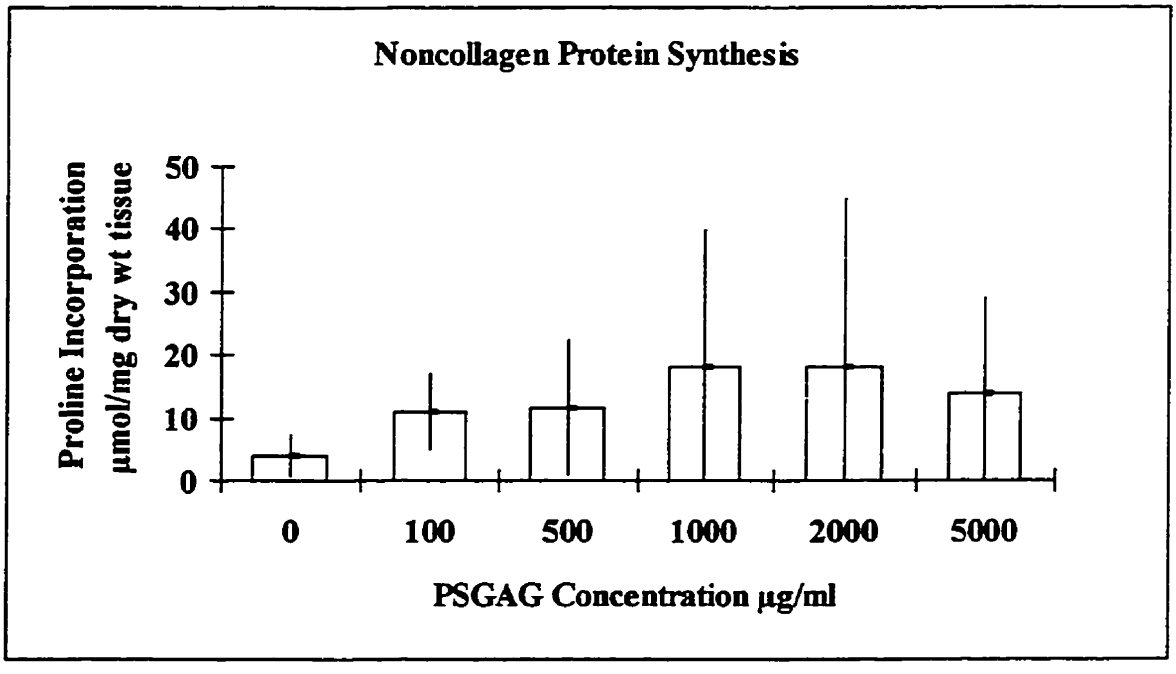

Figure 6.3: Noncollagen protein synthesis - Mean rate of proline incorporation into noncollagen protein per mg dry weight of equine SDFT by concentration of PSGAG in the culture medium (mean + sd µmol/mg dry weight of tendon).

Table 6.4: Noncollagen protein synthesis - Mean proline incorporation rate into noncollagen protein† per mg dry weight of equine SDFT by concentration of PSGAG in the medium.

PSGAG Concentration in Medium (μg/ml)	Proline Incorporation Mean ± SD (μmol/mg)	Statistical Groupings ($p < 0.05$)*	
0	4.04 ± 3.26	A	
100	10.76 ± 6.47		B
500	11.61 ± 10.61		B
1000	17.98 ± 21.71		B
2000	18.16 ± 26.51		B
5000	13.98 ± 14.97		B

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

6.4.4 PSGAG Treated Cultures - Glycosaminoglycans Synthesis

Mean total sulfate incorporation rates onto GAGs were significantly greater for the 5000 μg/ml, 2000 μg/ml, 1000 μg/ml and 500 μg/ml PSGAG treatment groups than the control group ($p < 0.01$) (table 6.5). The mean total sulfate incorporation rates were greater for the 2000 μg/ml and 1000 μg/ml than the 100 μg/ml PSGAG group ($p < 0.05$), lending some statistical support to the apparent dose related response observed in figure 6.4.

Table 6.5: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of PSGAG in the medium.

PSGAG Concentration in Medium (µg/ml)	Sulfate Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*		
0	44.13 ± 21.37	A		
100	63.49 ± 13.63	A	B	
500	86.53 ± 47.62		B	C
1000	117.75 ± 60.65			C
2000	115.47 ± 70.77			C
5000	89.17 ± 34.44		B	C

* Common letters denote that groups were not significantly different from each other

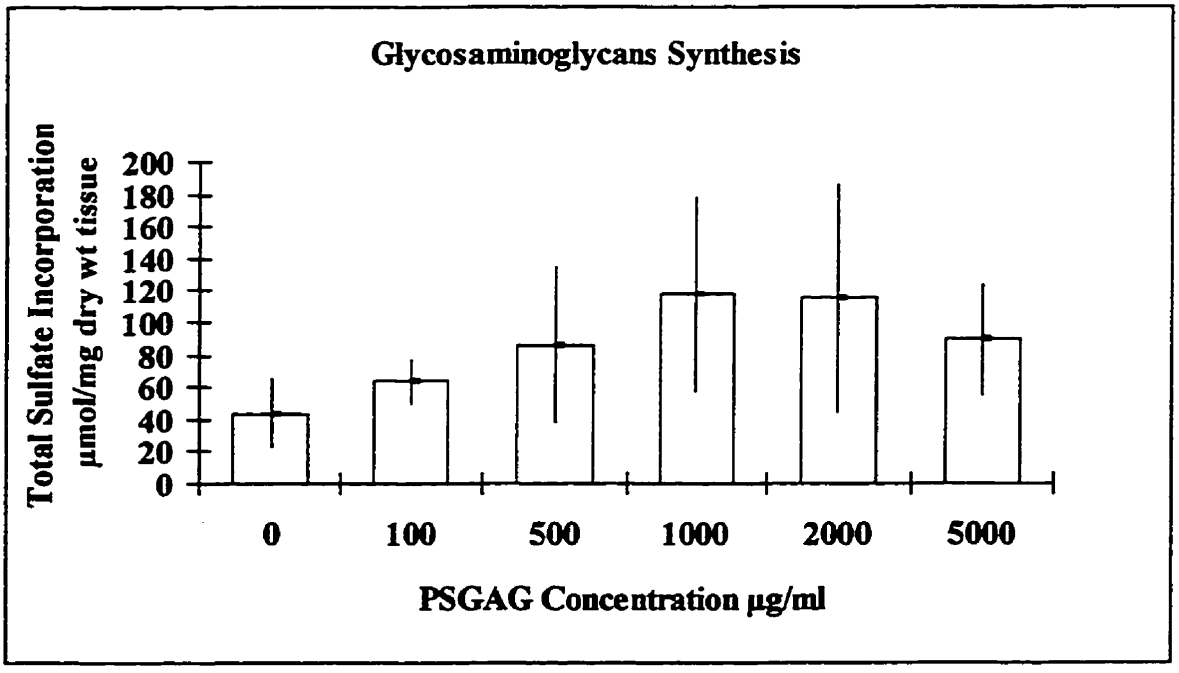

Figure 6.4: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of PSGAG in the culture medium (mean + sd µmol/mg dry weight of tendon).

6.4.5 PSGAG Treated Cultures - Tendon Explant Dry Weight

There were no significant differences in mean equine SDFT explant dry weights among PSGAG treatment and control groups (table 6.6, figure 6.5).

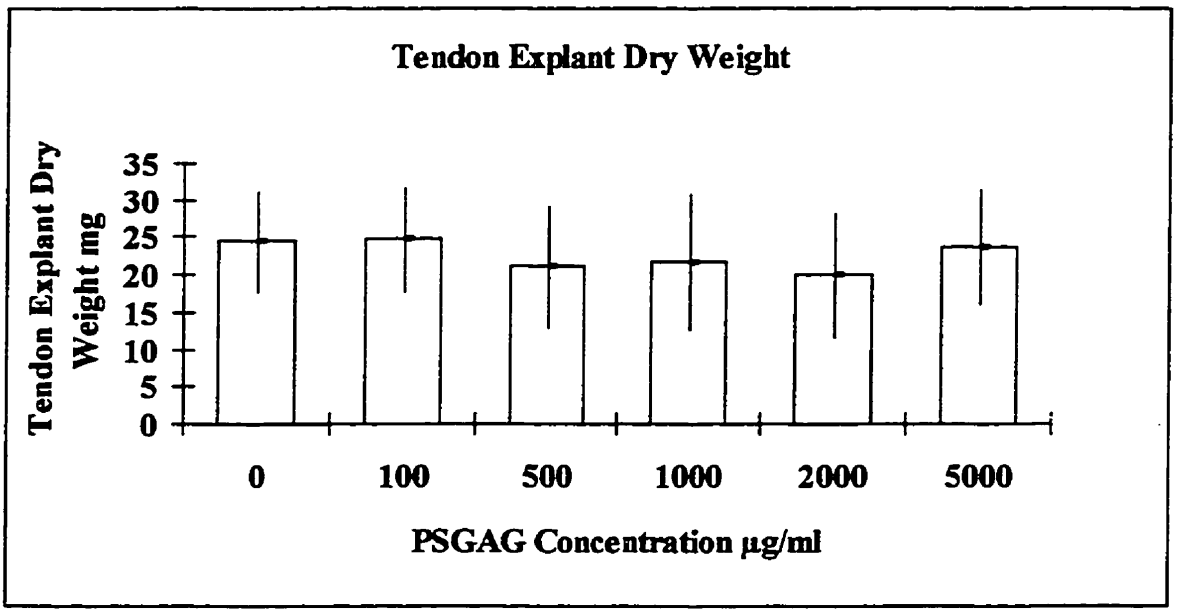

Figure 6.5: Tendon Explant Dry Weight - Mean equine SDFT explant dry weight by concentration of PSGAG in the culture medium (mean + sd mg dry weight of tendon).

Table 6.6: Tendon Explant Dry Weight - Mean tendon explant dry weight by concentration of PSGAG in the medium.

PSGAG Concentration in Medium (µg/ml)	Explant Dry Wt Mean ± SD mg	Statistical Grouping
0	24.47 ±6.67	A
100	24.75 ±6.99	A
500	21.10 ±8.11	A
1000	21.74 ±8.94	A
2000	19.90 ±8.35	A
5000	23.73 ±7.61	A

* Common letters denote that groups were not significantly different from each other.

6.4.6 rhIGF-I Treated Cultures - Total Protein Synthesis

Mean total proline incorporation rates were not significantly different among rhIGF-I treatment and control groups ($p > 0.10$), although there was a significant difference among the 500 ng/ml and 100 ng/ml rhIGF-I groups ($p < 0.05$) (table 6.7 and figure 6.6).

Table 6.7: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*	
0	72.06 ± 26.22	A	B
50	70.15 ± 7.48	A	B
100	69.96 ± 41.03		B
250	71.69 ± 16.70	A	B
500	78.78 ± 11.47	A	

*Common letters denote that groups were not significantly different from each other

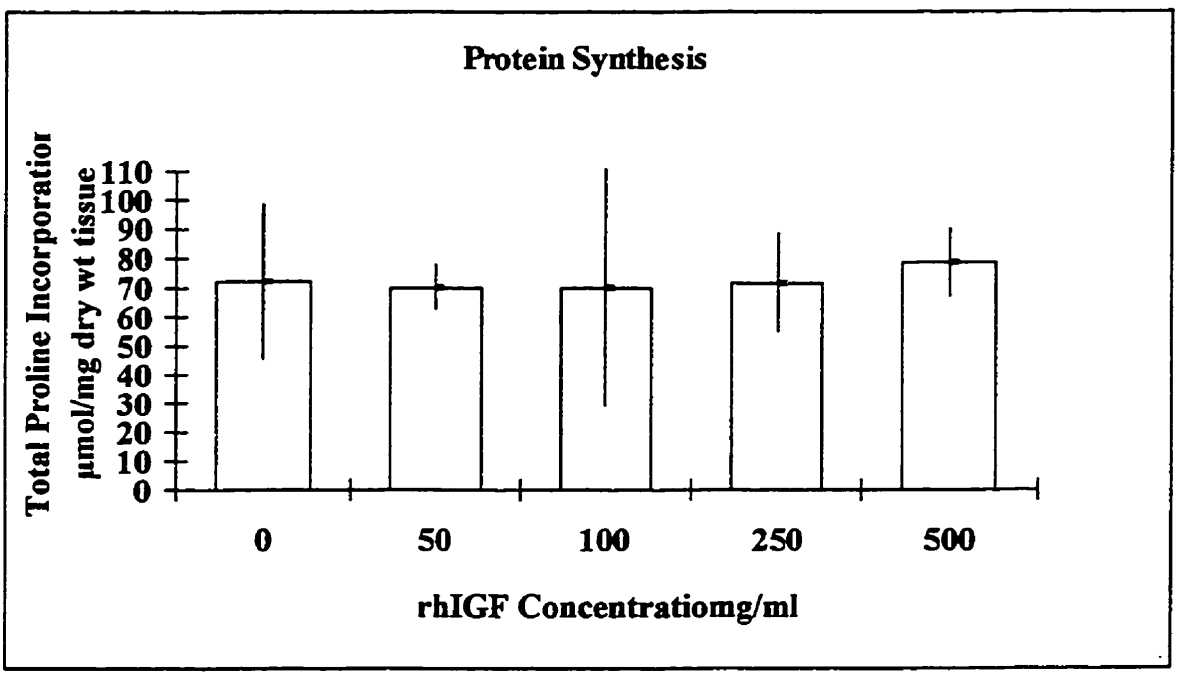

Figure 6.6: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd µmol/mg dry weight of tendon).

6.4.7 rhIGF-I Treated Cultures - Collagen Synthesis

Mean proline incorporation rate into collagen was significantly greater for the 500 ng/ml than the control group ($p < 0.05$) (figure 6.7, table 6.8).

Table 6.8: Collagen synthesis - Mean proline incorporation rate into collagen† per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*	
0	7.57 ± 7.35	A	
50	6.63 ± 3.68	A	B
100	4.38 ± 2.70	A	B
250	9.42 ± 4.65	A	B
500	8.97 ± 2.73		B

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

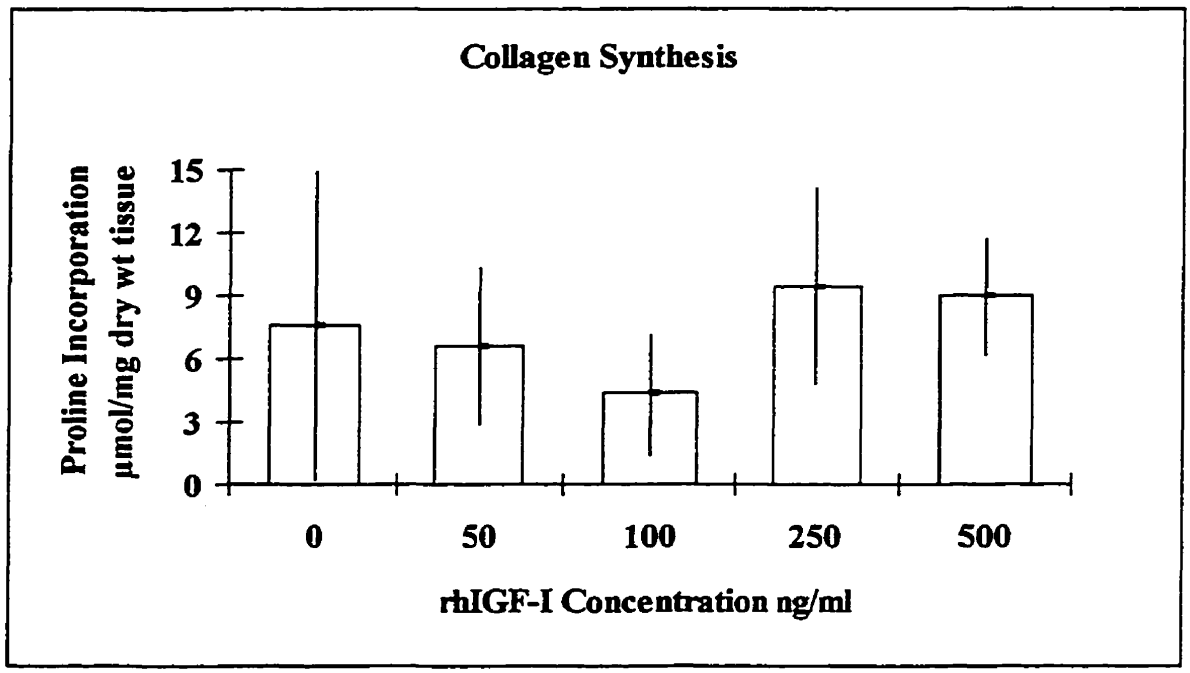

Figure 6.7: Collagen synthesis - Mean rate of proline incorporation into collagen (in the medium) per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd µmol/mg dry weight of tendon).

6.4.8 rhIGF-I Treated Cultures - Noncollagen Protein Synthesis

Mean proline incorporation rates into noncollagen protein were not significantly different among rhIGF-I treatment and control groups ($p > 0.10$) (table 6.9, figure 6.8).

Table 6.9: Noncollagen protein synthesis - Mean proline incorporation rate into noncollagen protein† per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*
0	4.04 ± 3.26	A
50	3.05 ± 1.66	A
100	3.16 ± 2.18	A
250	3.86 ± 4.64	A
500	2.81 ± 4.26	A

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

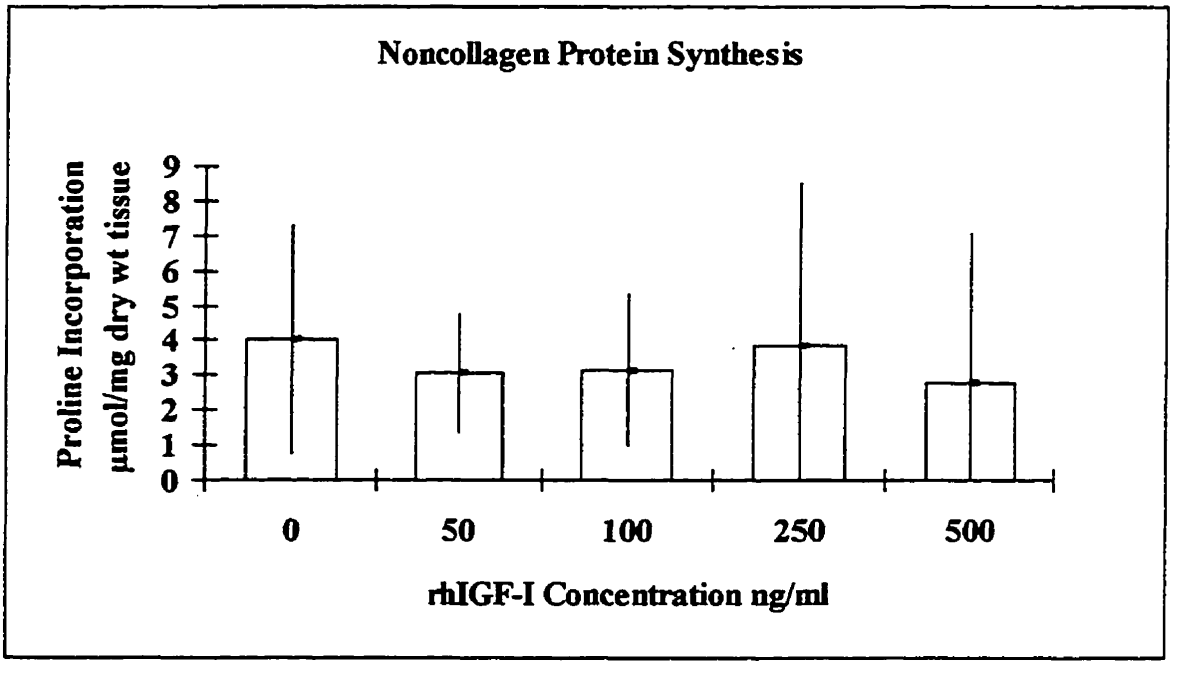

Figure 6.8: Noncollagen protein synthesis - Mean rate of proline incorporation into noncollagen protein per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd µmol/mg dry weight of tendon).

6.4.9 rhIGF-I Treated Cultures - Glycosaminoglycan Synthesis

Mean total sulfate incorporation rates onto glycosaminoglycans were not significantly different among rhIGF-I treatment and control groups ($p > 0.10$)(table 6.10, figure 6.9).

Table 6.10: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Sulfate Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*
0	44.13 ± 21.37	A
50	36.50 ± 5.81	A
100	40.29 ± 17.86	A
250	46.31 ± 15.28	A
500	43.53 ± 9.57	A

*Common letters denote that groups were not significantly different from each other.

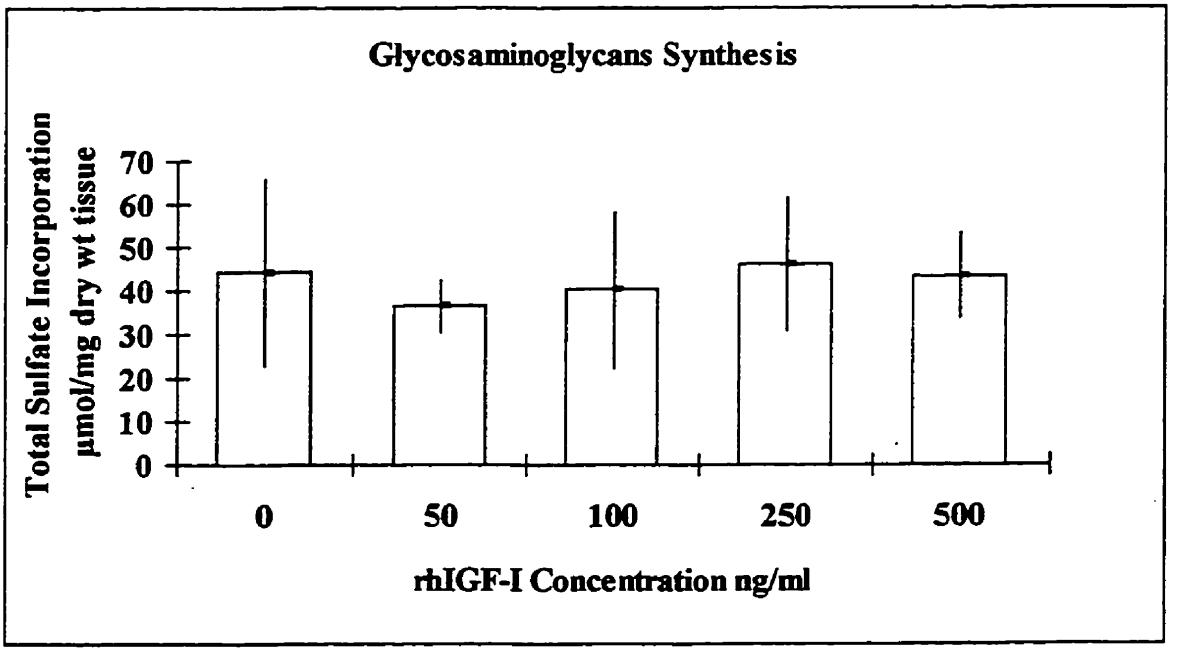

Figure 6.9: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the culture medium (mean + sd µmol/mg dry weight of tendon).

6.4.10 rhIGF-I Treated Cultures - Tendon Explant Dry Weight

There were no significant differences in mean equine SDFT explant dry weights among rhIGF-I treatment and control groups (table 6.11, figure 6.10).

Table 6.11: Tendon Explant Dry Weight - Mean tendon explant dry weight by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium ng/ml	Explant Dry Wt Mean ± SD mg	Statistical Grouping
0	24.47 ±6.67	A
50	26.65 ± 5.02	A
100	23.91 ± 6.51	A
250	23.49 ± 7.99	A
500	24.80 ± 7.09	A

* Common letters denote that groups were not significantly different from each other.

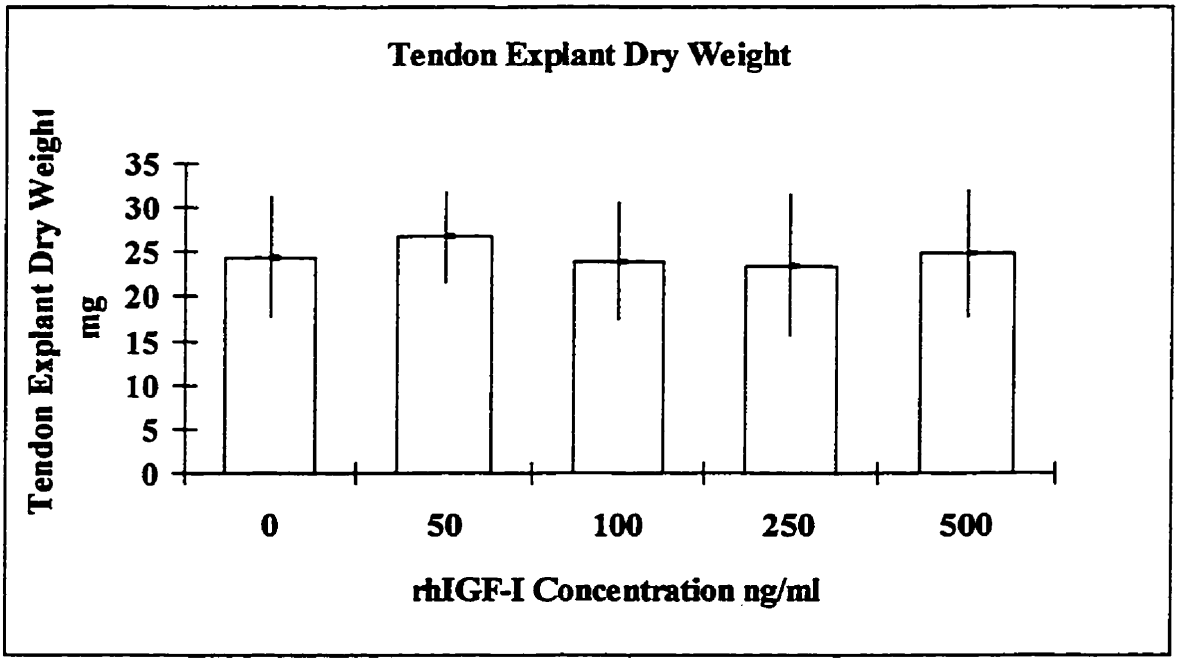

Figure 6.10: Tendon Explant Dry Weight - Mean equine SDFT explant dry weight by concentration of rhIGF-I in the culture medium (mean + sd mg dry weight of tendon).

6.5 Discussion

Although a small number of explants were excluded from statistical analysis, generally explant dry weight was distributed homogeneously among groups and cultures submitted for evaluation were mycoplasma free.

Significant increases in protein (both collagen and noncollagen protein) and glycosaminoglycan synthetic rates occurred for concentrations of PSGAG greater than 100 µg/ml, confirming the validity of performing a dose-response trial and increasing sample size (and thus power) for obtaining conclusive results and disproving the initial null hypothesis. A dose response was noted in the results, with stimulation of synthetic rates up to 1000 to 2000 µg/ml and an apparent decrease in response at higher doses (demonstrated with significance in glycosaminoglycans synthesis). Other studies have also demonstrated differences in response based on the concentration of PSGAG over a similar concentration range (Nethery *et al* 1992). The results from the current experiment validate this experimental approach, and resulted in a similar experiment being performed for the evaluation of NaHA.

There were three major limitations in the evaluation of synthetic responses to the PSGAG. Firstly, cell proliferation as determined by thymidine incorporation was not measured. It was initially planned to measure this variable, however failure of the isotope shipment to arrive prevented this determination, and delayed the treatment period from six to seven days. Cell proliferation was measured in experiment six. The second limitation is that nonsulfated GAGs (HA) were not measured. Since HA is an essential component in wound healing, it was also measured in experiment six. The third limitation of this experiment is that the mechanism of stimulation of synthetic rates was not elucidated. This limitation is beyond the scope of the experiments in this dissertation, but the potential mechanisms of stimulation will be mentioned in the general discussion section.

Although the doses of rhIGF-I used in this experiment were comparable to those of other studies only the rate of collagen synthesis was increased significantly at the 500 ng/ml concentration (Abrahamsson 1991; Murphy and Nixon 1997). As mentioned in

experiment three, equine SDFT explants may be less responsive than other tissues (Abrahamsson 1991). To confirm these results a further study was suggested, and cell proliferation and ECM synthesis were evaluated in experiment six.

6.6 Conclusions

1) Protein (collagen and noncollagen protein) and glycosaminoglycans synthetic rates were significantly increased in the presence of PSGAG at concentrations higher than 100 μg/ml.
2) The degree of response to PSGAG appeared to be dose related, with optimal concentration for increased synthesis at 1000 to 2000 μg/ml in the culture medium.
3) An increased rate of collagen synthesis but not total proline incorporation occurred in response to rhIGF-I at 500 ng/ml in the culture medium, but no other significant effects were observed and the response was not demonstrably linearly dose related over the concentration range tested.

CHAPTER SEVEN

7.0 EXPERIMENT 5 : EVALUATION OF THE EFFECT OF DOSE ON THE METABOLIC AND PROLIFERATIVE RESPONSES OF EQUINE SDFT EXPLANTS TO SODIUM HYALURONATE AND RECOMBINANT HUMAN IGF-I

7.1 Introduction

The protocol followed in experiment four produced results which clearly demonstrated the anabolic effects of exogenous PSGAG on explants of the equine SDFT, and validated the pre-treatment culture period of 18 days. In experiments two and three it appeared that the response to treatment with HA differed depending upon the pre-treatment stabilization period (as reported for PSGAG), with an apparently inhibitory response in shorter term cultures, and no demonstrable response in longer term cultures. Statistical power calculations using the results of experiments two and three confirmed the need for larger treatment groups for comparison of control and treated explants. As demonstrated in for PSGAG, the effect of different concentrations may be an important determinant of the degree of response, if any, of the SDFT explant cultures to NaHA.

In experiment four, the addition of rhIGF-I (at doses ranging from 50 to 500 ng/ml) to explant cultures of the equine SDFT resulted in the stimulation of only sulfated GAG synthesis at 500 ng/ml. The lack of response in protein synthesis (collagen and noncollagen protein) and the low magnitude of the response to 500 ng/ml rhIGF-I contrast with the large dose dependent responses reported in the rabbit and the horse (Abrahamsson *et al* 1991a&b; Murphy and Nixon 1997). However, as previously stated, the equine cultures were treated with rhIGF-I in the presence of 5% FBS which has high concentrations of growth factors including IGF-I (Jayme 1990; Riley 1994). Unlike these previous studies in which the

response to rhIGF-I was dependent upon the concentration of the factor in the medium, a dose effect was not demonstrated in experiment four. It is possible that the doses of rhIGF-I used were too high or too low, or that the equine SDFT is non-responsive to the rhIGF-I.

This aim of this experiment is to test the hypotheses that 1) The addition of exogenous NaHA at different concentrations to explant cultures of the equine SDFT will not significantly affect rates of cell proliferation or sulfated GAG and protein synthesis compared to a control group, and 2) The addition of rhIGF-I at different concentrations to explant cultures of the equine SDFT will not significantly affect rates of cell proliferation or sulfated GAG and protein synthesis compared to a control group of cultures.

7.2 Objectives

1) To determine the effects of NaHA on the rates of cell proliferation and the synthesis of protein and sulfated GAG in equine SDFT explant cultures at different concentrations.
2) To determine the effects of rhIGF-I on the rates of cell proliferation and the synthesis of protein and sulfated GAG in equine SDFT explant cultures at different concentrations.

7.3 Materials and Methods

7.3.1 Experimental Animal

A two-year-old male Quarter horse cross (~ 1.5 m at the shoulder), untrained and free from clinical evidence of tendon injury, was purchased from the Saskatoon Auction Mart

7.3.2 Establishment of Equine SDFT Explant Organ Cultures

The tensile portions of the forelimb SDFT's were harvested and explant cultures prepared as per the previously developed protocol. Cultures were maintained on rollers at 36.5±0.5°C for the duration of the experiment (26 days) and the media (RPMI 1640

containing proline, 10% DHS, 100 µg/ml ascorbate) was changed every 72 h. After an 18 day pre-treatment period, cultures were treated with different doses of either NaHA (Hyonate®, Bayer Incorporated, ONT., Canada) or rhIGF-I (Upstate Biotechnology Inc., NY., USA) for six days prior to radio labeling (table 7.1). A total of 246 cultures were obtained; six were randomly selected and tested for mycoplasma at day 18.

Table 7.1: Treatment groups for NaHA and rhIGF-I dose response trials.

Treatment	Concentration µg/ml	Number of Explants	Treatment	Concentration ng/ml	Number of Explants
Control*	0	40	Control*	0	40
NaHA†	50	40	rhIGF-I‡	5	10
NaHA†	500	40	rhIGF-I‡	10	10
NaHA†	1000	40	rhIGF-I‡	25	10
NaHA†	2000	40	rhIGF-I‡	100	10

†Hyonate® 20 mg/ml, Bayer Incorporated, ONT., Canada, ‡rhIGF-I, Upstate Biotechnology Inc., NY., USA, *common control group for rhIGF-I and NaHA.

7.3.3 Radiolabeling of Equine SDFT Explant Organ Cultures

Cultures were radiolabeled [^{35}S-sulfate (20 µCi/culture) and L-[2,3,4,5-^{3}H] proline (20 µCi/culture)] for 24 h at the end of the treatment period (day 24) and chase incubated for 12 hours with 2 ml of serum free medium. Cultures were also labeled with 1 µCi/culture of methyl-C^{14}-thymidine (SPA 55.0 mCi/mmol) for 24 h as an index of cell proliferation.

7.3.4 Determination of Radioactive Isotope Incorporation Rates

Determination of Incorporation Rates of Radioactive Isotopes by Tendon Explants

The preparation and scintillation counting of the tendon explants and of the labeled media, as well as the calculation of incorporation rates were performed as described in Appendix III.

Aliquots of culture medium were precipitated in 0.15% DOCA and 72%TCA, incubated for 10 minutes at room temperature, centrifuged for 30 minutes at 3300 g and the supernatant decanted. Each protein pellet was digested in 6M HCl for 24 hours at 110^{0}C and then the pH adjusted to 9.0. Aliquots of 40 or 45 µl were derivatized with DABS-Cl and then Hyp and Pro separated by RP-HPLC. Fractions corresponding to the Hyp and Pro peaks were collected, scintillation cocktail added and the fractions counted on a beta counter. The ratio of the counts and a formula accounting for the relative concentrations of Hyp and Pro in collagen was used to determine molar rates per mg dry weight of proline incorporation into collagen and noncollagen protein precipitated from the medium.

7.3.5 Statistical Analysis

All results were tabulated and their distribution and variances determined. Extreme outliers and negative values were excluded from analyses (5 explants). Protein and sulfated GAG synthesis and cell proliferation were compared by parametric one way ANOVA except where the requirements of parametric testing were not met. In these cases the Kruskal-Wallis non-parametric ANOVA was used and a means comparison performed among treatment groups. Significance level was set at $p < 0.05$, with a trend recognized at $p < 0.10$.

7.4 Results

Cultures submitted were free of mycoplasma and bacterial contamination.

7.4.1 NaHA Treated Cultures - Total Protein Synthesis

Mean total proline incorporation rates were greater for the 2000 µg/ml ($p < 0.001$) and 1000 µg/ml ($p < 0.10$) NaHA treatment groups than the control group (table 7.2). All

other NaHA groups had incorporation rates significantly greater than that of the 50 µg/ml group ($p < 0.02$), and the 2000 µg/ml group had a higher rate than the 1000 µg/ml group ($p < 0.02$), supporting the apparent dose related response observed in figure 7.1.

Table 7.2: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of NaHA in the medium.

NaHA Concentration in Medium (µg/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.02$)*		
0	57.45 ± 28.73	A	B	
50	42.53 ± 23.37	A		
500	62.84 ± 26.14		B	
1000	68.44 ± 22.88		B	
2000	83.00 ± 36.38			C

* Common letters denote that groups were not significantly different from each other.

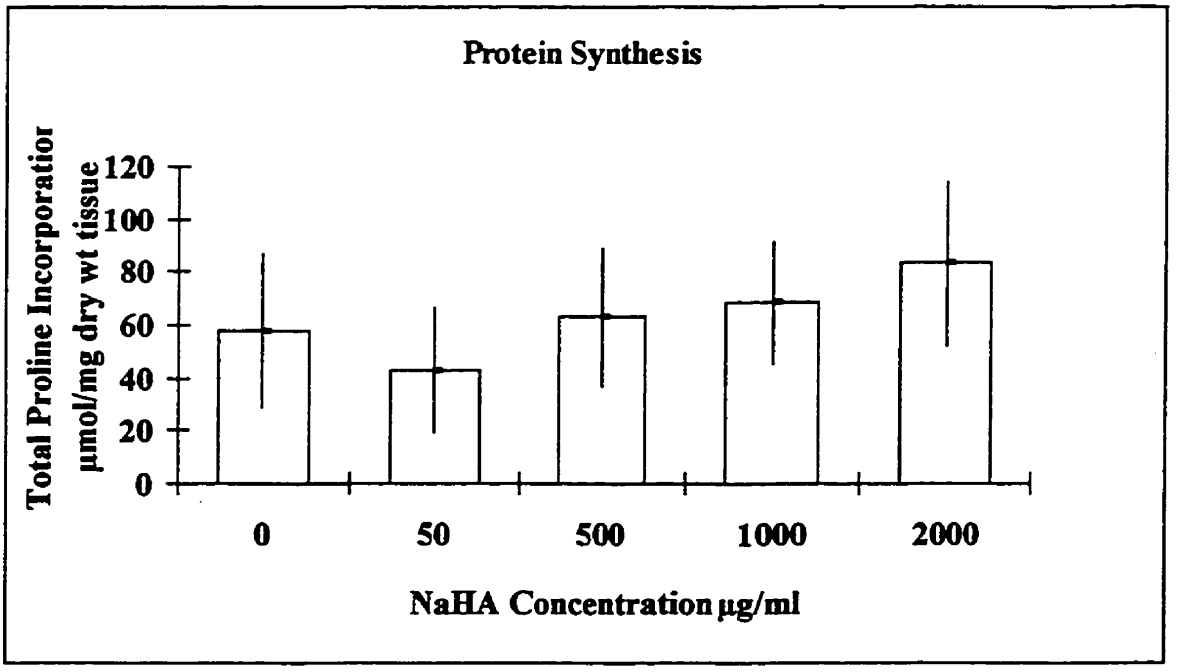

Figure 7.1: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of NaHA in the medium (mean + sd µmol/mg dry weight of tendon).

7.4.2 NaHA Treated Cultures - Collagen Synthesis

Mean proline incorporation rates into collagen were greater for the 2000 μg/ml ($p < 0.10$) and 1000 μg/ml ($p < 0.02$) NaHA treatment groups than the control (table 7.3). The rate of collagen synthesis was greater in the 2000 μg/ml and 1000 μg/ml than the 500 μg/ml and 50 μg/ml NaHA treatment groups ($p < 0.02$) (figure 7.2).

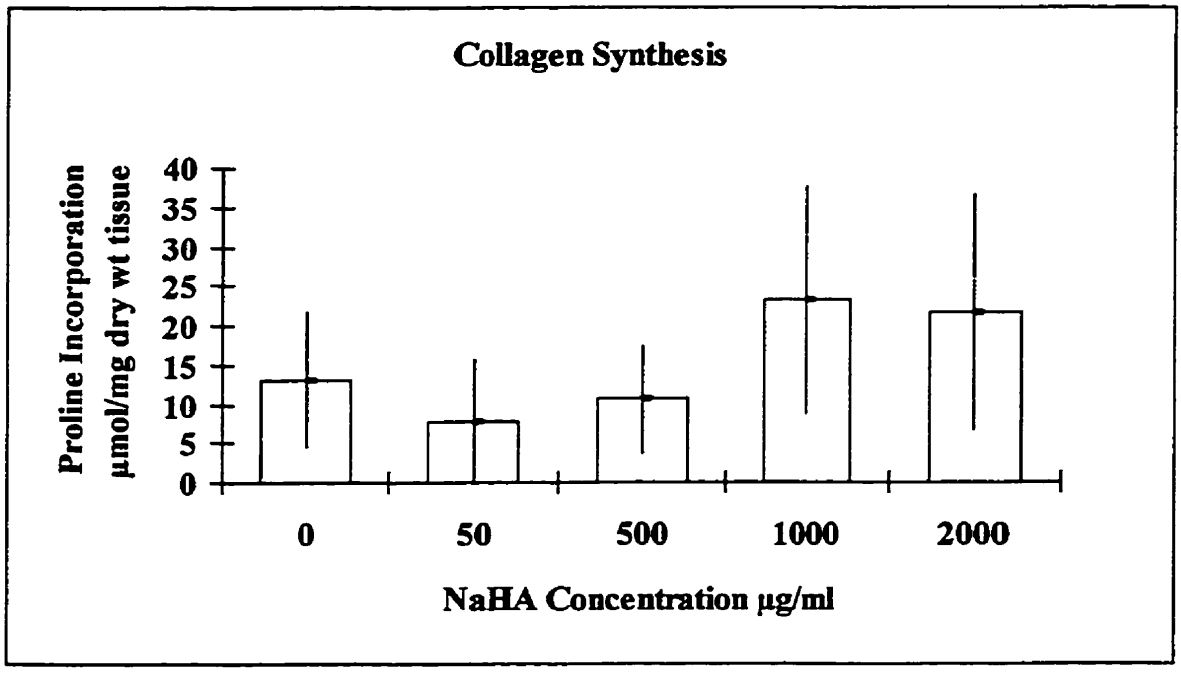

Figure 7.2: Collagen synthesis - Mean rate of proline incorporation into collagen (in the medium) per mg dry weight of equine SDFT by concentration of NaHA in the medium (mean + sd μmol/mg dry weight of tendon).

Table 7.3: Collagen synthesis - Mean proline incorporation rate into collagen† per mg dry weight of equine SDFT by concentration of NaHA in the medium.

NaHA Concentration in Medium (µg/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*		
0	13.09 ± 8.51		B	C
50	7.64 ± 8.20			C
500	10.57 ± 6.89			C
1000	23.25 ± 14.46	A		
2000	21.62 ± 15.00	A	B	

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

7.4.3 NaHA Treated Cultures - Noncollagen Protein Synthesis

Mean proline incorporation rates into noncollagen protein were not significantly different among NaHA treatment and the control groups ($p > 0.05$)(table 7.4). The mean noncollagen protein incorporation rate for the 2000 µg/ml group was greater than those of the 1000 µg/ml and 50 µg/ml NaHA groups ($p < 0.05$)(figure 7.3).

Table 7.4: Noncollagen protein synthesis - Mean proline incorporation rate into noncollagen protein† per mg dry weight of equine SDFT by concentration of NaHA in the medium.

NaHA Concentration in Medium (µg/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.02$)*	
0	22.69 ± 12.97	A	B
50	16.91 ± 8.30		B
500	24.57 ± 15.89	A	B
1000	16.36 ± 11.77		B
2000	32.74 ± 16.33	A	

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

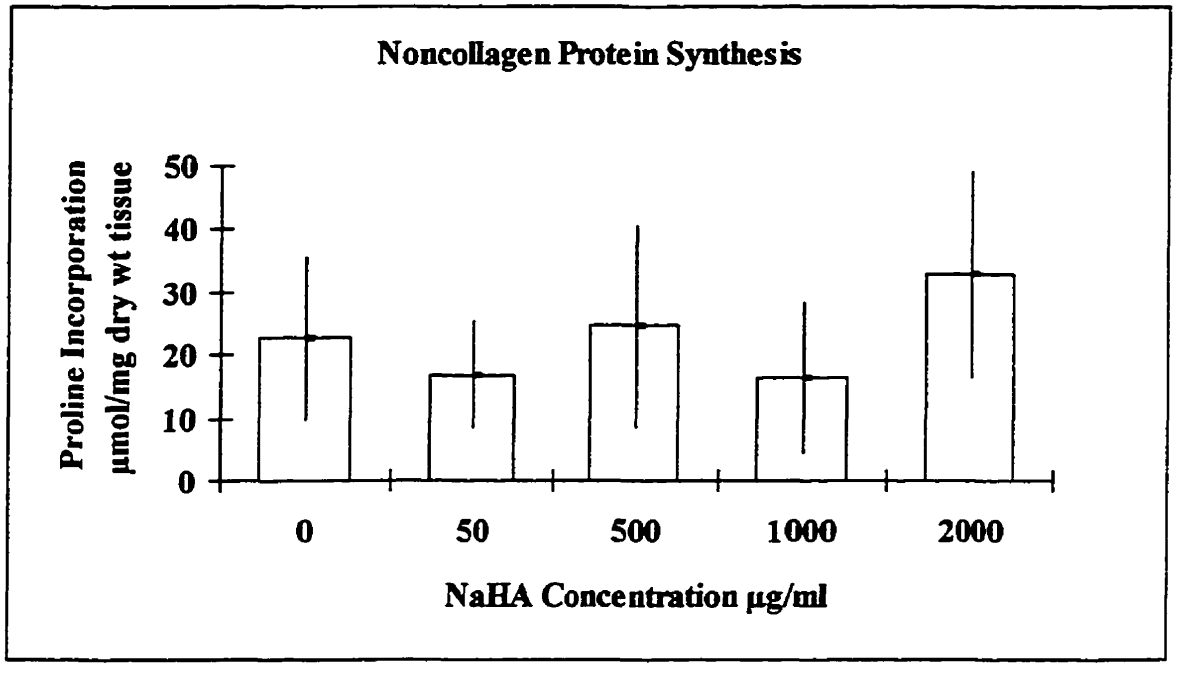

Figure 7.3: Noncollagen protein synthesis - Mean rate of proline incorporation into noncollagen protein per mg dry weight of equine SDFT by concentration of NaHA in the medium (mean + sd µmol/mg dry weight of tendon).

7.4.4 NaHA Treated Cultures - Glycosaminoglycans Synthesis

Mean total sulfate incorporation rates onto GAGs were significantly greater for the 2000 µg/ml than the 500 µg/ml, 50 µg/ml NaHA treatment groups and the control group ($p < 0.05$) (table 7.5). The mean total sulfate incorporation rates were greater for the 1000 µg/ml and than the 50 µg/ml NaHA group ($p < 0.05$), lending some statistical support to the apparent dose-related response observed in figure 7.4.

Table 7.5: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of NaHA in the medium.

NaHA Concentration in Medium (µg/ml)	Sulfate Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*		
0	162.65 ± 80.50	A	B	
50	120.64 ± 65.75	A		
500	167.43 ± 71.27		B	
1000	176.17 ± 60.14		B	C
2000	213.30 ± 79.88			C

* Common letters denote that groups were not significantly different from each other

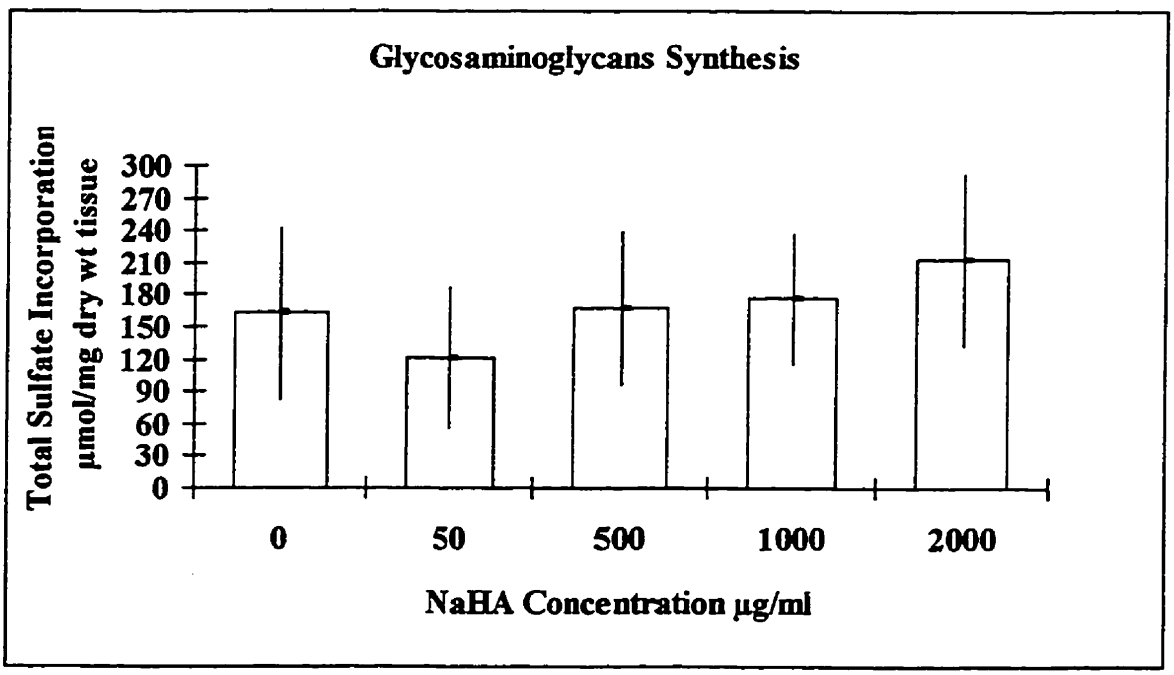

Figure 7.4: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of NaHA in the medium (mean + sd µmol/mg dry weight of tendon).

7.4.5 NaHA Treated Cultures - Cell Proliferation

The rate of cell proliferation was lower in the 50 μg/ml NaHA group than the control and 2000 μg/ml NaHA groups ($p < 0.05$) (table 7.6, figure 7.5).

Table 7.6: Cell proliferation - Mean thymidine incorporation rate per mg dry weight of equine SDFT by concentration of NaHA in the medium.

NaHA Concentration in Medium (μg/ml)	Thymidine Incorporation Mean ± SD (μmol/mg)	Statistical Groupings ($p < 0.05$)*	
0	13.62 ± 3.80	A	
50	10.97 ± 5.13		B
500	11.99 ± 3.83	A	B
1000	12.32 ± 3.82	A	B
2000	13.16 ± 3.42	A	

* Common letters denote that groups were not significantly different from each other

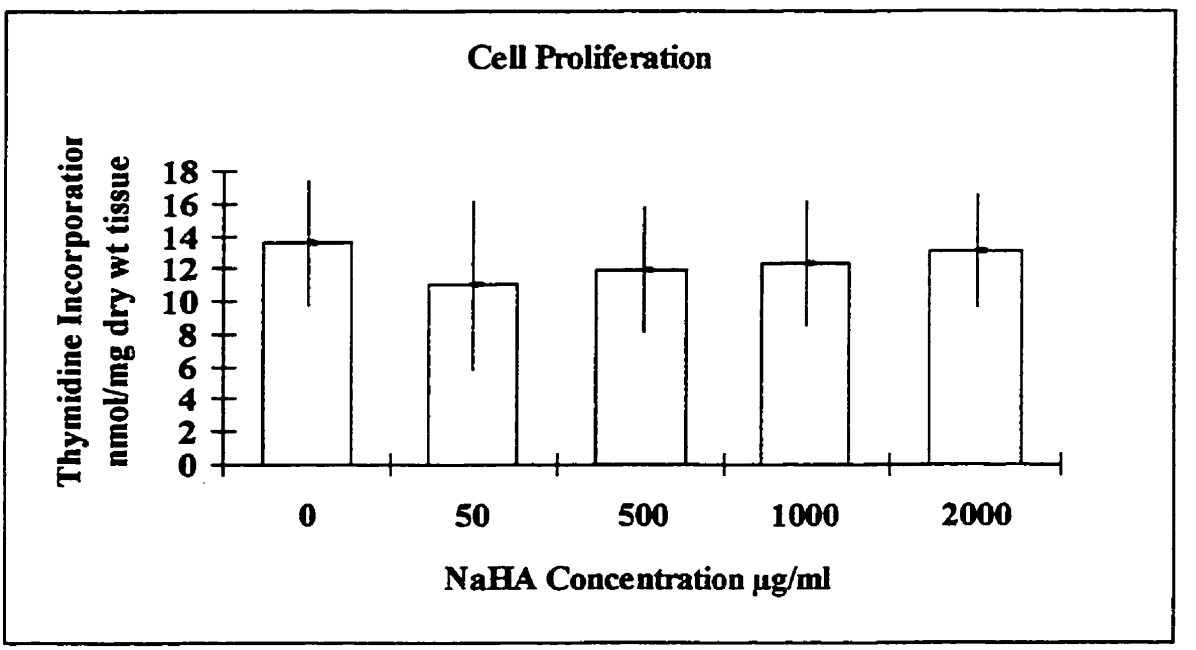

Figure 7.5: Cell Proliferation - Mean thymidine incorporation rate per mg dry weight equine SDFT by concentration of NaHA in the medium (mean + sd μmol/mg dry weight of tendon).

7.4.6 NaHA Treated Cultures - Tendon Explant Dry Weight

There were no significant differences in mean equine SDFT explant dry weights among NaHA treatment and control groups (table 7.7, figure 7.6).

Table 7.7: Tendon Explant Dry Weight - Mean tendon explant dry weight by concentration of NaHA in the medium.

NaHA Concentration in Medium (µg/ml)	Explant Dry Wt Mean ± SD mg	Statistical Grouping
0	11.37 ± 3.79	A
50	11.13 ± 3.36	A
500	11.77 ± 2.69	A
1000	10.92 ± 2.82	A
2000	11.53 ± 3.46	A

* Common letters denote that groups were not significantly different from each other.

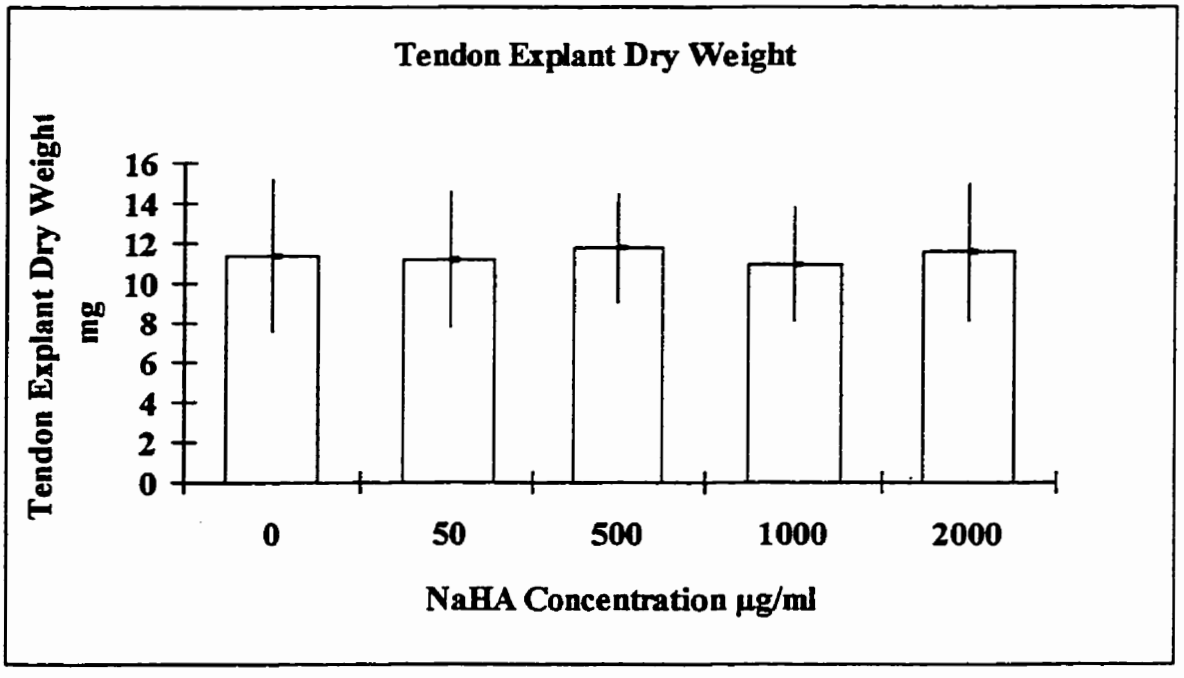

Figure 7.6: Tendon Explant Dry Weight - Mean equine SDFT explant dry weight by concentration of NaHA in the medium (mean + sd mg dry weight of tendon).

7.4.7 rhIGF-I Treated Cultures - Total Protein Synthesis

Mean total proline incorporation rates were significantly greater for all rhIGF-I treatment groups than the control group ($p < 0.01$) (table 7.8, figure 7.7).

Table 7.8: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.01$)*
0	57.45 ± 28.73	A
5	101.81 ± 40.45	B
10	128.94 ± 42.12	B
25	129.83 ± 43.21	B
100	123.78 ± 57.21	B

* Common letters denote that groups were not significantly different from each other.

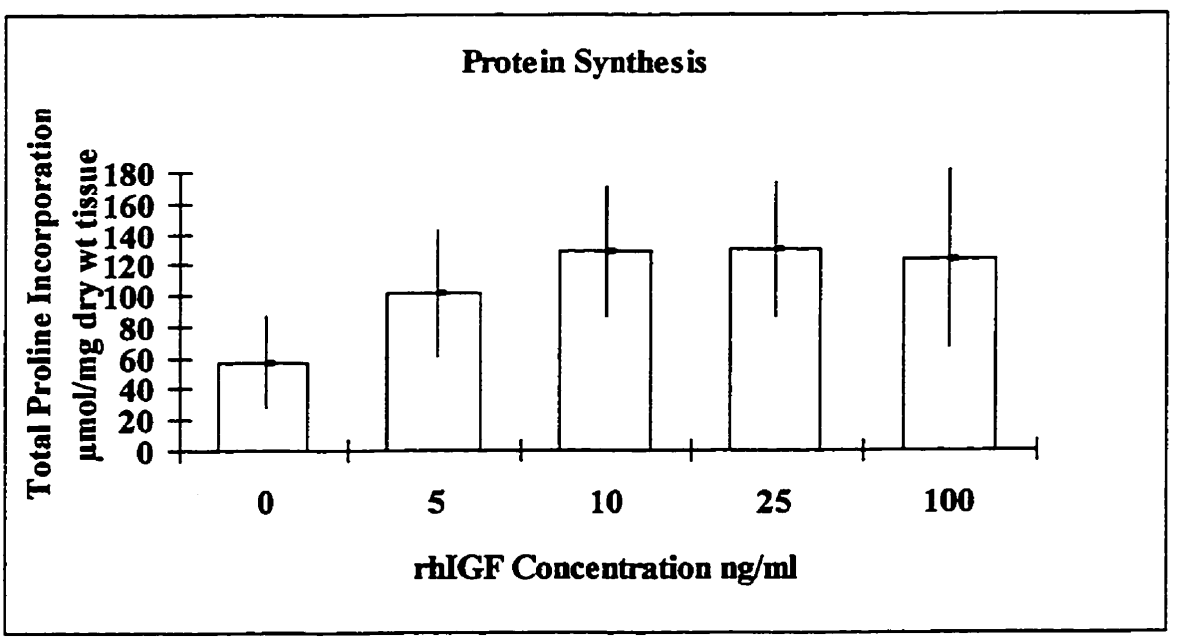

Figure 7.7: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd µmol/mg dry weight of tendon).

7.4.8 rhIGF-I Treated Cultures - Collagen Synthesis

Mean proline incorporation rates into collagen were significantly greater in all except the 5 ng/ml rhIGF-I groups than the control group($p < 0.01$)(figure 7.8, table 7.9)

Table 7.9: Collagen synthesis - Mean proline incorporation rate into collagen† per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)*	
0	18.04 ± 15.20	A	
5	24.84 ± 12.41	A	B
10	31.19 ± 13.35		B
25	38.56 ± 16.67		B
100	36.27 ± 28.44		B

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

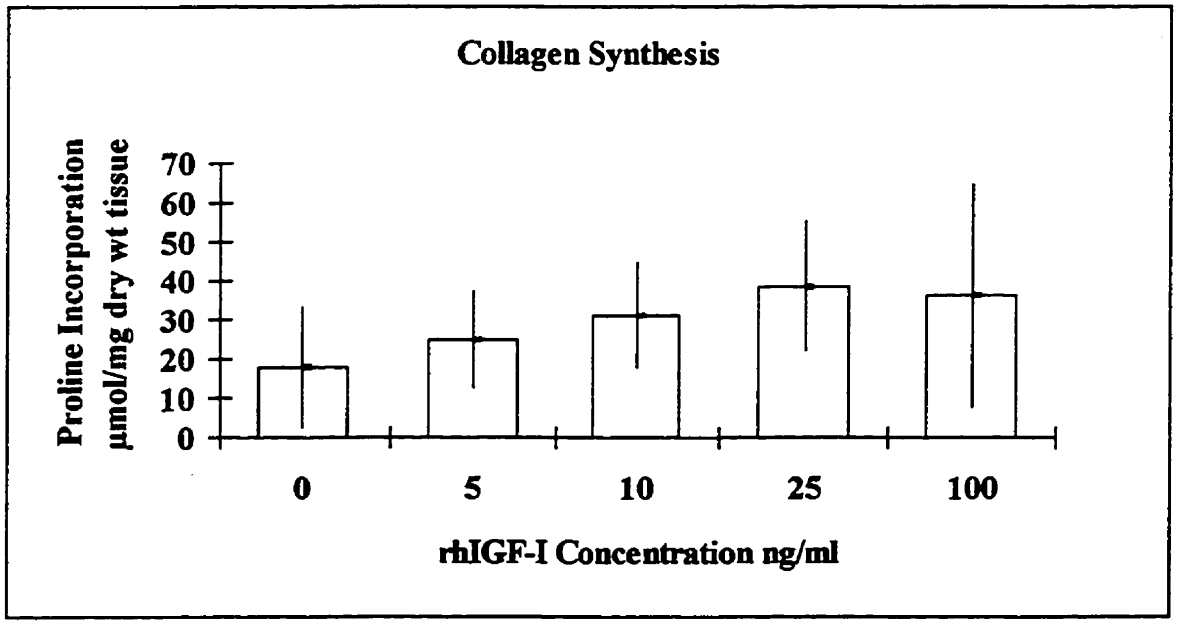

Figure 7.8: Collagen synthesis - Mean rate of proline incorporation into collagen (in the medium) per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd µmol/mg dry weight of tendon).

7.4.9 rhIGF-I Treated Cultures - Noncollagen Protein Synthesis

Mean proline incorporation rates into noncollagen protein were significantly greater in all rhIGF-I groups than the control group ($p < 0.01$) (figure 7.9, table 7.10).

Table 7.10: Noncollagen protein synthesis[†]-Mean proline incorporation rate into noncollagen protein per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium

rhIGF-I Concentration in Medium (ng/ml)	Proline Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.05$)[•]
0	24.52 ± 15.63	A
5	52.72 ± 25.64	B
10	67.58 ± 27.83	B
25	61.59 ± 27.62	B
100	58.55 ± 34.45	B

[†]Determined by RP-HPLC of digested protein precipitate from culture medium; [•]common letters denote that groups were not significantly different from each other.

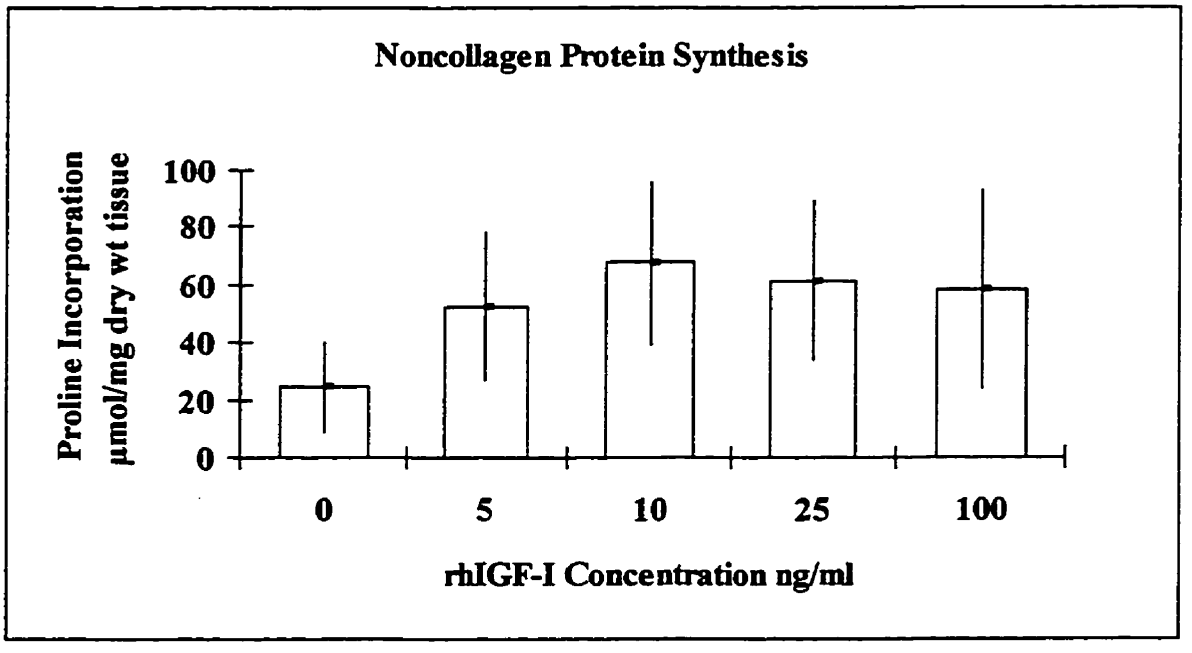

Figure 7.9: Noncollagen protein synthesis - Mean rate of proline incorporation into noncollagen protein per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd µmol/mg dry weight of tendon).

7.4.10 rhIGF-I Treated Cultures - Glycosaminoglycan Synthesis

Mean total sulfate incorporation rates onto glycosaminoglycans were not significantly different among rhIGF-I treatment and control groups ($p > 0.10$)(table 7.11, figure 7.10).

Table 7.11: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Sulfate Incorporation Mean ± SD (µmol/mg)	Statistical Groupings ($p < 0.01$)*
0	162.65 ± 80.50	A
5	289.74 ± 118.36	B
10	359.55 ± 115.02	B
25	370.25 ± 121.28	B
100	352.69 ± 160.31	B

* Common letters denote that groups were not significantly different from each other

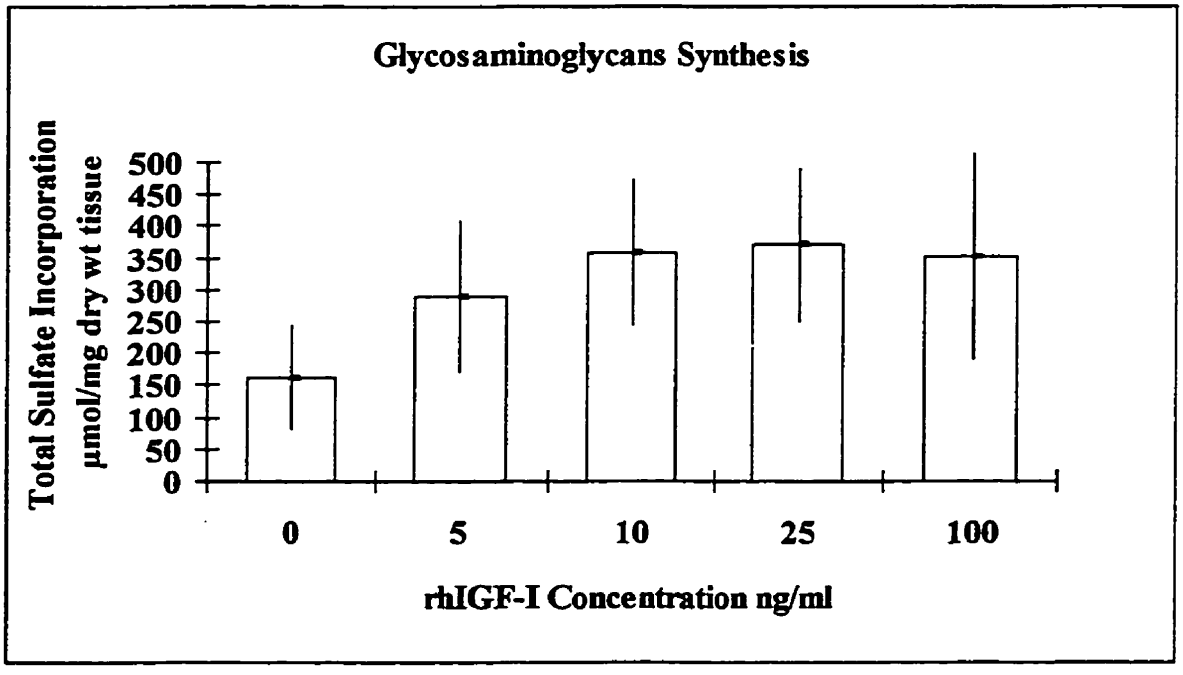

Figure 7.10: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd µmol/mg dry weight of tendon).

7.4.11 rhIGF-I Treated Cultures - Cell Proliferation

Mean rates of cell proliferation as determined by thymidine incorporation were significantly greater in all except the 5 ng/ml rhIGF-I treatment groups than the control group($p < 0.05$)(figure 7.11, table 7.12)

Table 7.12: Cell Proliferation - Mean thymidine incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium (ng/ml)	Thymidine Incorporation Mean ± SD (μmol/mg)	Statistical Groupings ($p < 0.05$)*	
0	13.62 ± 3.80	A	
5	16.21 ± 3.48	A	B
10	19.12 ± 3.58		B
25	19.25 ± 4.39		B
100	18.50 ± 4.51		B

* Common letters denote that groups were not significantly different from each other..

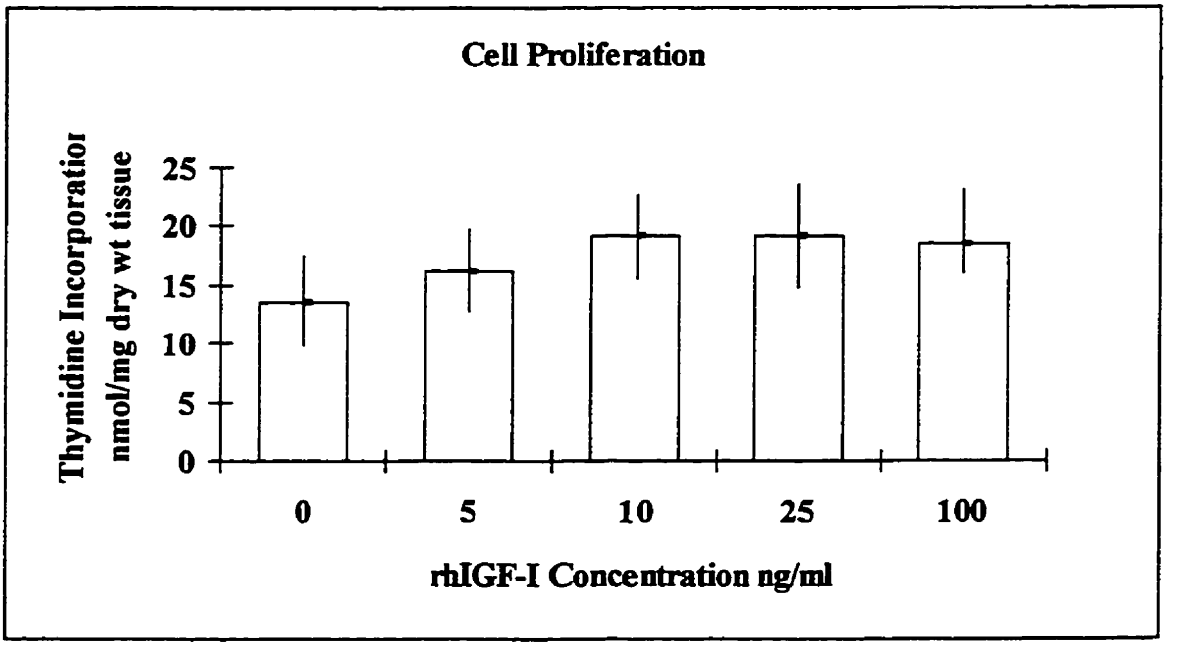

Figure 7.11: Cell Proliferation - Mean thymidine incorporation rate per mg dry weight of equine SDFT by concentration of rhIGF-I in the medium (mean + sd nmol/mg dry weight of tendon).

7.4.12 rhIGF-I Treated Cultures - Tendon Explant Dry Weight

There were no significant differences in mean equine SDFT explant dry weights among rhIGF-I treatment and control groups (table 7.13, figure 7.12).

Table 7.13: Tendon Explant Dry Weight - Mean tendon explant dry weight by concentration of rhIGF-I in the medium.

rhIGF-I Concentration in Medium ng/ml	Explant Dry Wt Mean ± SD mg	Statistical Grouping
0	11.37 ± 3.79	A
5	11.59 ± 3.00	A
10	10.32 ± 2.43	A
25	10.51 ± 3.00	A
100	12.79 ± 6.09	A

* Common letters denote that groups were not significantly different from each other.

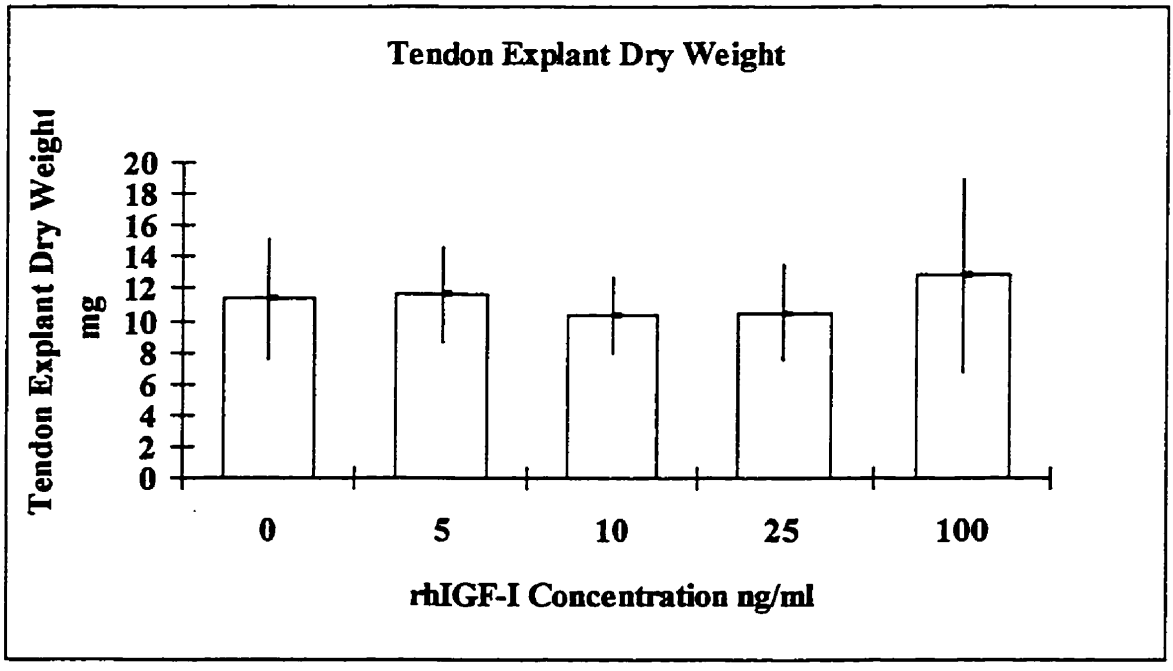

Figure 7.12: Tendon Explant Dry Weight - Mean equine SDFT explant dry weight by concentration of rhIGF-I in the medium (mean + sd mg dry weight of tendon).

7.5 Discussion

Explant dry weights were distributed homogeneously among groups and cultures submitted for evaluation were mycoplasma free.

Increases in rates of total proline incorporation into protein, collagen synthesis and sulfated GAG synthesis were observed for the higher doses of NaHA. There were differences in protein and GAG synthesis between high, intermediate and low concentrations of NaHA, indicative of a dose dependent response. However, the latter two groups were not significantly different from the control group. The response of the 50 μg/ml group appeared to indicate inhibition of synthetic responses at low concentrations. The upper concentration of NaHA used (2000 μg/ml) was determined by the commercial preparation itself which at 20 mg/ml, represented 20% of the volume of the medium (which was corrected to ensure equal nutrient and serum concentrations among groups). Higher doses could be evaluated if more concentrated forms of the product became available, with caution given to the effect such preparations may have on medium osmolality. Cell proliferation did not appear to be affected by NaHA in this experiment. The anabolic responses demonstrated for NaHA require experimental confirmation (experiment six).

All synthetic rates were increased in the presence of rhIGF-I. This occurred in contrast to the previous experiments, all of which examined the response to rhIGF-I to doses greater than 100 ng/ml. The horse used in this experiment was slightly younger than that used in experiment four, and may have contributed to the response observed. However, although the increase from 25 ng/ml to 100 ng/ml was four-fold, a concomitant four-fold increase in synthesis did not occur. This latter fact suggests that even higher doses are unlikely to increase the response observed, or may even be inhibitory.

7.6 Conclusions

1) Protein (collagen but not noncollagen protein) and glycosaminoglycans synthetic rates, but not cell proliferation, were significantly increased in the presence of NaHA at concentrations of 1000 μg/ml or 2000 μg/ml.

2) The degree of response to NaHA appeared to be dose related, however values for concentrations lower than 1000 μg/ml were not significantly different from the controls.

3) The responses to NaHA at 50 μg/ml may be inhibition of protein and GAG synthesis.

4) An increase in rates of protein synthesis (collagen and noncollagen protein), sulfated GAG synthesis, and cell proliferation occurred in response to rhIGF-I at doses greater than or greater than and equal to 5 ng/ml in the culture medium.

5) The response to rhIGF-I was not demonstrably dose related over the concentration range tested.

CHAPTER EIGHT

8.0 EXPERIMENT 6 : COMPARISON OF THE METABOLIC AND CELL PROLIFERATION RESPONSES OF EQUINE SDFT EXPLANTS TO POLYSULFATED GLYCOSAMINOGLYCANS AND SODIUM HYALURONATE

8.1 Introduction

The protocol followed in experiment four and five produced results which demonstrated anabolic effects in response to exogenous PSGAG and NaHA respectively on explants of the equine SDFT. However, previous work in the horse has demonstrated variation in biochemical composition and in the biological response to tissue culture (Riley 1994; Bailey *et al* unpublished data). Therefore reproduction of the results were considered necessary to confirm the observations made in the chapters six and seven.

In the rabbit PSGAG, chondroitin 6-sulfate, chondroitin 4-sulfate and other individual sulfated GAGs have been shown to increase HA synthesis of the knee synovial membrane (Nishika *et al* 1985). In experiment four it was indicated that cell proliferation and synthesis of HA should be measured in order to obtain further information on the effect of PSGAG. The latter parameter can be readily measured by radioimetric assay of HA in the culture medium (Tengblad 1980). However for NaHA treated cultures it would be necessary to use radiolabeled NaHA or glucosamine to distinguish the exogenous HA from the newly synthesized HA, followed by dialysis and chromatographic separation (Nishika *et al* 1985). The development of such a protocol is beyond the scope of this study so HA measurement be limited to radiometric assay of the medium from PSGAG treatment and control groups.

The response of the explants to PSGAG appeared to be more marked than that of the explants exposed to NaHA, as was the apparent dose - response relationship over the

comparable portions of the dose ranges. Critical evaluation of these observations requires comparison of the two drugs at similar levels. Determining molarity of the formulations was beyond the scope of the current study. Therefore NaHA and PSGAG were compared on the basis of weight per unit volume at a high end dose. The suspected inhibitory effect of low concentrations of HA (50 μg/ml) also required further investigation with an increase in samples size per group to ensure adequate statistical power.

This aim of this experiment is to test the hypotheses that 1) There are no differences in the rates of protein and GAG synthesis, or cell proliferation between equine SDFT explant cultures exposed to exogenous NaHA or PSGAG at equivalent concentrations, and 2) The addition of NaHA at high and low concentrations to explant cultures of the equine SDFT will not significantly affect rates of cell proliferation, sulfated GAG and protein synthesis compared to a control group of cultures.

8.2 Objectives

1) To compare the effects of NaHA (1000 μg/ml) and PSGAG (1000 μg/ml) to each other and to a control group (0 μg/ml) on the rates of cell proliferation, protein (collagen and noncollagen protein) and GAG (sulfated and nonsulfated HA) synthesis of equine SDFT explant cultures at different NaHA concentrations.

2) To determine the effects of NaHA on the rates of cell proliferation, protein (collagen and noncollagen protein) and sulfated GAG synthesis of equine SDFT explant cultures at 50 μg/ml and 1000 μg/ml.

8.3 Materials and Methods

8.3.1 Experimental Animal

A two-year-old female Thoroughbred (~ 1.5 m at the shoulder), untrained and free from clinical evidence of tendon injury, was purchased from a local farm.

8.3.2 Establishment of Equine SDFT Explant Organ Cultures

The tensile portions of the forelimb SDFT's were harvested and explant cultures prepared as per the previously developed protocol. Cultures were maintained on rollers at 36.5±0.5°C for the duration of the experiment (26 days) and the media (RPMI 1640 containing proline, with 10% DHS and 100 µg/ml ascorbate) changed every 72 h unless otherwise indicated. After a pre-treatment period of 18 days, cultures were exposed to either NaHA or PSGAG for six days prior to radio labeling (table 8.1). A total of 246 cultures were obtained; six were randomly selected and tested for mycoplasma at day 18.

Table 8.1: Treatment groups for comparison of NaHA and PSGAG.

Treatment	Concentration µg/ml	Number of Explants
Control	0	70
NaHA†	50	50
NaHA†	1000	50
PSGAG‡	1000	70

†Hyonate® 20 mg/ml, Bayer Incorporated, ONT., Canada. ‡Adequan® 100 mg/ml, Shirley, NY., USA.

8.3.3 Radiolabeling of Equine SDFT Explant Organ Cultures

Cultures were radiolabeled with ^{35}S-sulfate (20 µCi/culture, SPA 1497 Ci/mmol) and L-[2,3,4,5-^{3}H] proline (20 µCi/culture, SPA 114.0 Ci/mmol) for 24 h at the end of the treatment period (day 24) and chase incubated for 12 hours as per the established protocol with 2 ml of serum free medium. Cultures were also labeled with 1 µCi/culture of methyl-C^{14}-thymidine (SPA 55.0 mCi/mmol) as an index of cell proliferation for 24 h. Twenty control and 20 PSGAG explants were not radiolabeled but were harvested and stored for possible later analyses.

8.3.4 Determination of Radioactive Isotope Incorporation Rates

Determination of Incorporation Rates of Radioactive Isotopes by Tendon Explants

The preparation and scintillation counting of the tendon explants and of the labeled media, as well as the calculation of incorporation rates were performed as described in Appendix III.

Pre-column Derivatization and RP-HPLC

Aliquots of culture medium were precipitated in 0.15% DOCA and 72%TCA, incubated for 10 minutes at room temperature, centrifuged for 30 minutes at 3300 g and the supernatant decanted. Each protein pellet was digested in 6M HCl for 24 hours at 110°C and then the pH adjusted to 9.0. Aliquots of 40 or 45 µl were derivatized with DABS-Cl and then Hyp and Pro separated by RP-HPLC. Fractions corresponding to the Hyp and Pro peaks were collected, scintillation cocktail added and the fractions counted on a beta counter. The ratio of the counts and a formula accounting for the relative concentrations of Hyp and Pro in collagen was used to determine molar rates per mg dry weight of proline incorporation into collagen and noncollagen protein precipitated from the medium.

8.3.5 Determination of Rates of Hyaluronate Synthesis

A commercial radiometric assay kit was used for determination of concentrations of HA in the serum free medium (Pharmacia HA Test, Pharmacia AB, Sweden). Briefly, 100 µl aliquots of PSGAG, control and standard HA solutions were added to 200 µl of hyaluronic acid binding protein (HABP-^{125}I) in duplicate. Following an hour at 4°C, the mixture was incubated with 100 µl of HA-Sepharose at 4°C for an hour. The liquid was decanted and the remaining radioactivity measured in a gamma counter. The response is inversely proportional to the concentration of the HA. A standard curve was constructed, and the HA concentration of PSGAG and control samples determined from this curve.

8.3.6 Statistical Analysis

All results were tabulated and their distribution and variances determined. Extreme outliers and negative values were excluded from analyses (3 explants). Data were compared by parametric one-way ANOVA unless one of the conditions for parametric testing were not met. In this instance, the Kruskal-Wallis non-parametric ANOVA was used and a means comparison performed among treatment groups. Significance level was set at $p < 0.05$, with a trend recognized at $p < 0.10$.

8.4 Results

Cultures were free of mycoplasma, and bacterial contamination did not occur.

8.4.1 Total Protein Synthesis

Mean total proline incorporation rates were greater for the PSGAG group than all other groups ($p < 0.01$). The mean total proline incorporation rate for 1000 µg/ml NaHA ($p < 0.01$) and 50 µg/ml NaHA ($p < 0.02$) treatment groups were greater than that of the control group but not significantly different from each other (table 8.2, figure 8.1).

Table 8.2: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by treatment group.

Treatment Group		Proline Incorporation Mean ± SD µmol/mg	Statistical Groupings ($p < 0.02$)*		
Control	(0 µg/ml)	32.84 ± 7.90	A		
NaHA	(50 µg/ml)	36.75 ± 8.71		B	
NaHA	(1000 µg/ml)	38.44 ± 6.23		B	
PSGAG	(1000 µg/ml)	43.26 ± 6.94			C

*Common letters denote that groups were not significantly different from each other.

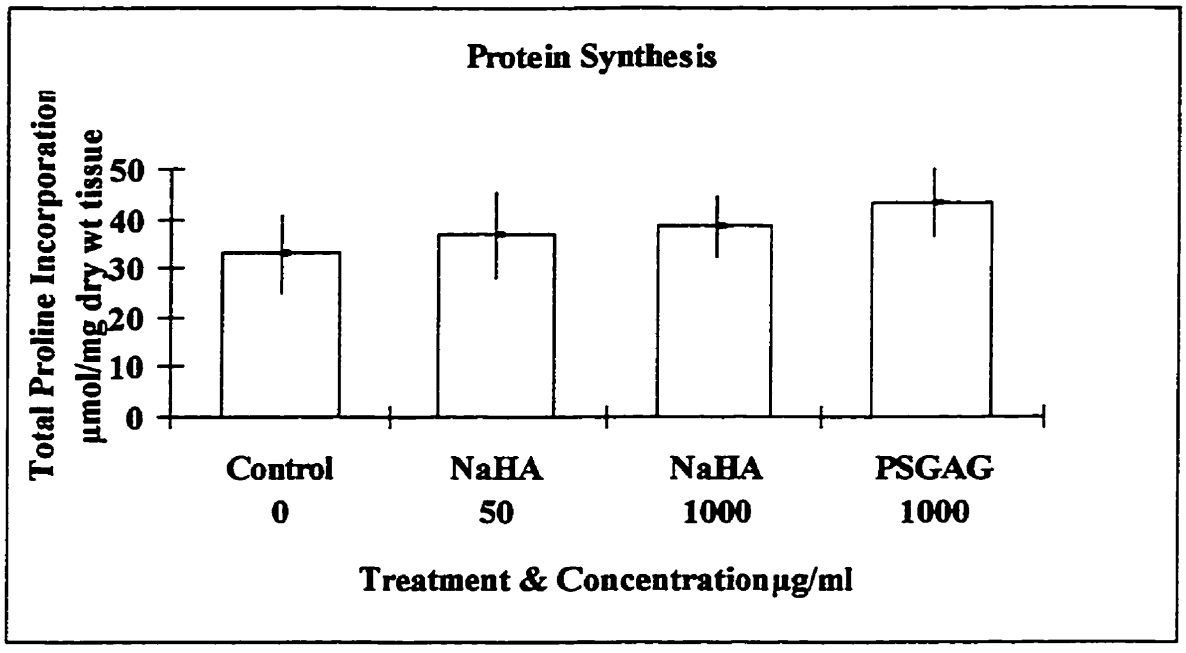

Figure 8.1: Protein synthesis - Mean total proline incorporation rate per mg dry weight of equine SDFT by treatment group (mean + sd µmol/mg dry weight of tendon).

8.4.2 Collagen Synthesis

Mean proline incorporation rates into collagen were significantly greater for the PSGAG and 50 µg/ml NaHA groups ($p < 0.01$), and tended to be greater in the 1000 µg/ml NaHA group ($p < 0.10$) than the control group (table 8.3). The rate of collagen synthesis was greater in the PSGAG group than the 1000 µg/ml NaHA group ($p < 0.05$) (figure 8.2).

Table 8.3: Collagen synthesis - Mean proline incorporation rate into collagen† per mg dry weight of equine SDFT by treatment group.

Treatment Group		Proline Incorporation Mean ± SD µmol/mg	Statistical Groupings ($p < 0.05$)*		
Control	(0 µg/ml)	3.34 ± 1.98	A		
NaHA	(1000 µg/ml)	4.12 ± 1.71	A	B	
NaHA	(50 µg/ml)	4.90 ± 3.22		B	C
PSGAG	(1000 µg/ml)	5.47 ± 2.31			C

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

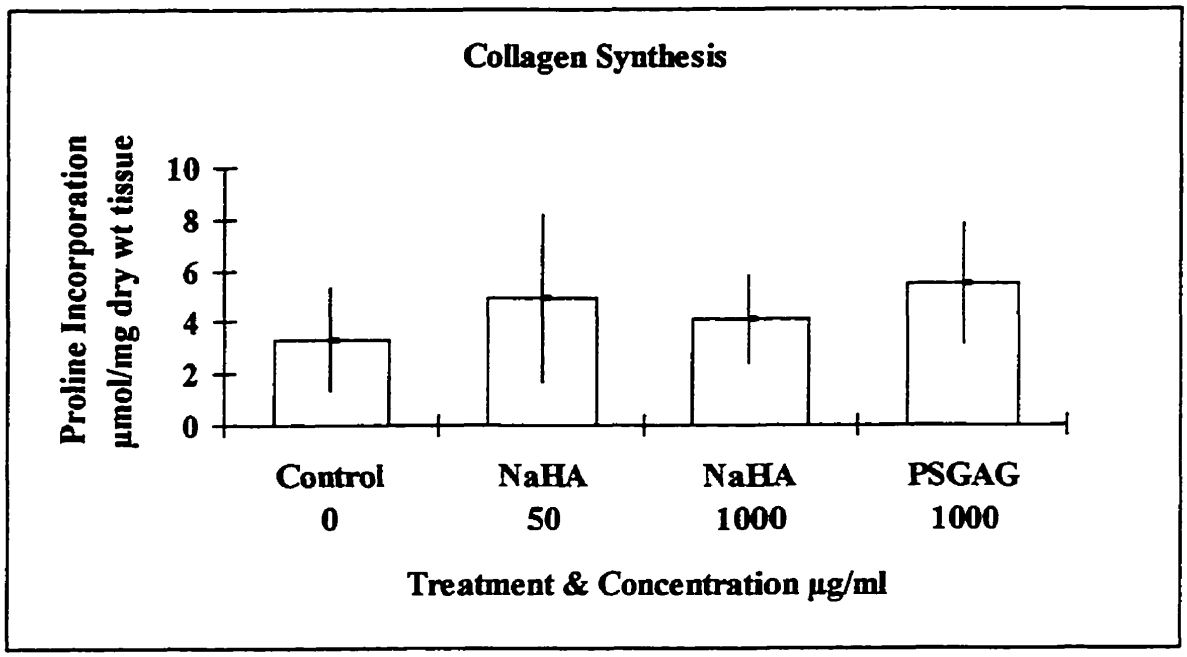

Figure 8.2: Collagen synthesis - Mean rate of proline incorporation into collagen (in the medium) per mg dry weight of equine SDFT by treatment group (mean + sd µmol/mg dry weight of tendon).

8.4.3 Noncollagen Protein Synthesis

Mean proline incorporation rates into noncollagen protein were significantly greater for the PSGAG group than the control group ($p < 0.05$), but there were no other significant differences among groups (table 8.4, figure 8.3).

Table 8.4: Noncollagen protein synthesis - Mean proline incorporation rate into noncollagen protein† per mg dry weight of equine SDFT by treatment group.

Treatment Group		Proline Incorporation Mean ± SD µmol/mg	Statistical Groupings ($p < 0.05$)*	
Control	(0 µg/ml)	6.22 ± 2.60	A	
NaHA	(50 µg/ml)	7.15 ± 3.56	A	B
NaHA	(1000 µg/ml)	7.19 ± 2.92	A	B
PSGAG	(1000 µg/ml)	7.96 ± 3.99		B

†Determined by RP-HPLC of digested protein precipitate from culture medium; *common letters denote that groups were not significantly different from each other.

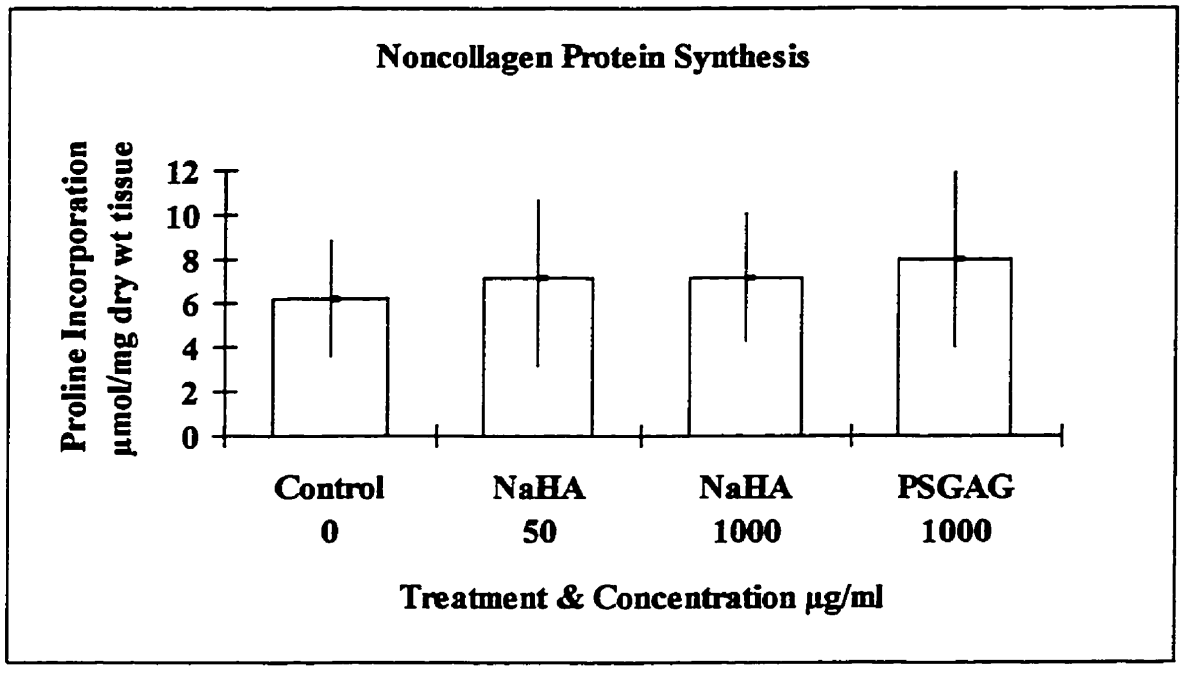

Figure 8.3: Noncollagen protein synthesis - Mean rate of proline incorporation into noncollagen protein per mg dry weight of equine SDFT by treatment group (mean + sd µmol/mg dry weight of tendon).

8.4.4 Sulfated Glycosaminoglycans Synthesis

Mean total sulfate incorporation rates onto sulfated GAGs were significantly greater for PSGAG than all other groups ($p < 0.01$), and for the 1000 µg/ml NaHA group than the 50 µg/ml NaHA and control groups ($p < 0.01$) (table 8.5, figure 8.4).

Table 8.5: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by treatment group.

Treatment Group		Sulfate Incorporation Mean ± SD µmol/mg	Statistical Groupings ($p < 0.01$)*
Control	(0 µg/ml)	71.17 ± 18.76	A
NaHA	(50 µg/ml)	76.60 ± 22.02	A
NaHA	(1000 µg/ml)	91.22 ± 15.88	B
PSGAG	(1000 µg/ml)	102.15 ± 19.89	C

*Common letters denote that groups were not significantly different from each other

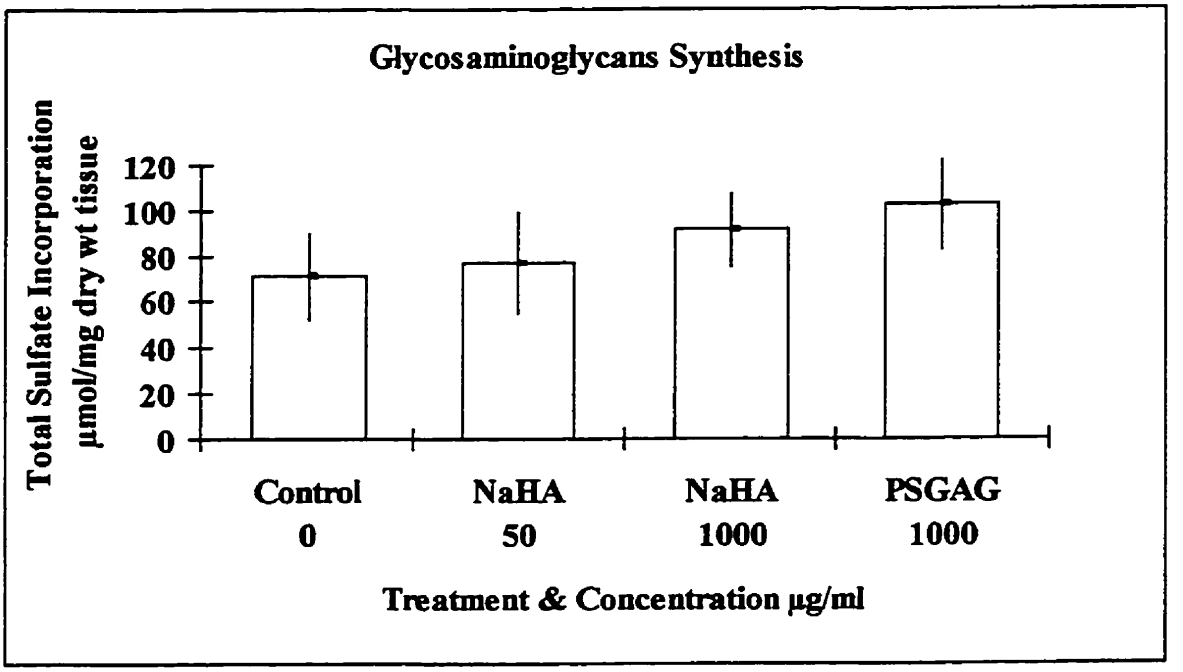

Figure 8.4: Glycosaminoglycans synthesis - Mean total sulfate incorporation rate per mg dry weight of equine SDFT by treatment group (mean + sd µmol/mg dry weight of tendon).

8.4.5 Hyaluronate Synthesis

The rate of HA synthesis was significantly reduced for the PSGAG group compared to the control group ($p < 0.001$)(table 8.6, figure 8.5).

Table 8.6: Hyaluronate synthesis - Mean rate of hyaluronate synthesis rate per mg dry weight of equine SDFT by treatment group.

Treatment Group		Hyaluronate Synthesis Mean ± SD nmol/mg	Statistical Groupings ($p < 0.001$)*	
Control	(0 µg/ml)	6.46 ± 2.46	A	
PSGAG	(1000 µg/ml)	3.49 ± 1.94		B

* Letters denote that groups were significantly different from each other

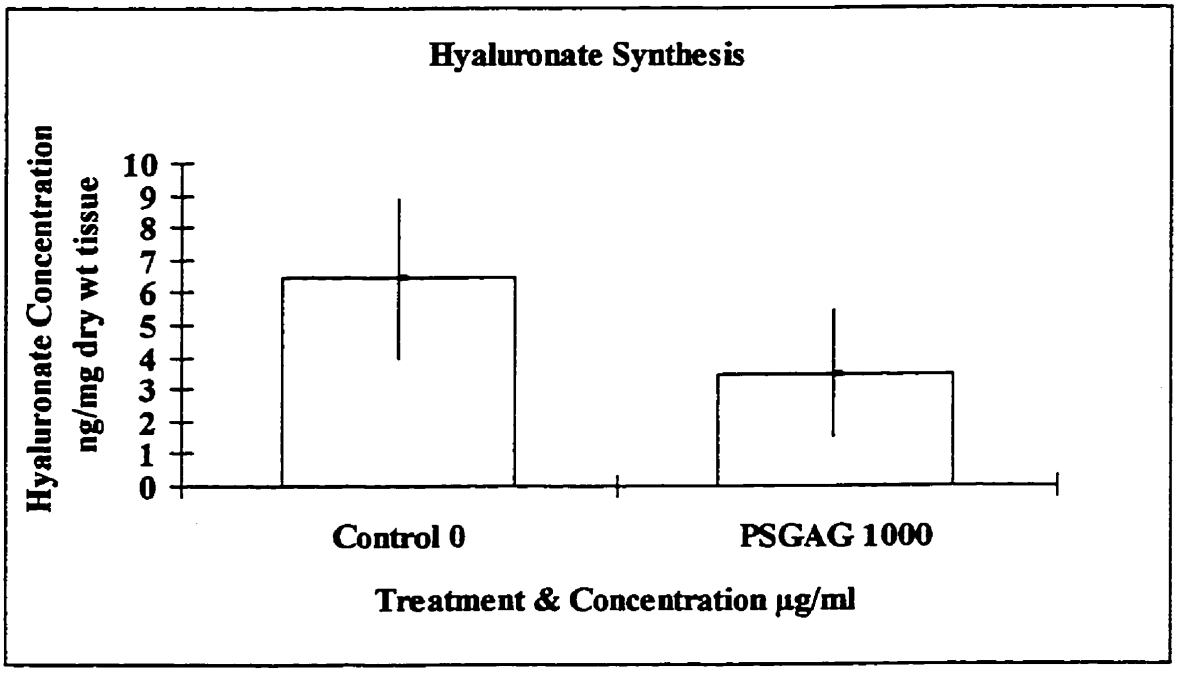

Figure 8.5: Hyaluronate Synthesis - Mean rate of HA synthesis rate per mg dry weight of equine SDFT by treatment group (mean + sd µmol/mg dry weight of tendon)

8.4.6 Cell Proliferation

The rates of cell proliferation were significantly greater for the PSGAG group than all other groups ($p < 0.01$), and for the 1000 µg/ml NaHA group than the 50 µg/ml NaHA group but not the control group when differences in dry weight were controlled for (table 8.7, figure 8.6).

Table 8.7: Cell proliferation - Mean thymidine incorporation rate per mg dry weight of equine SDFT by treatment group.

Treatment Group		Thymidine Incorporation Mean ± SD nmol/mg	Statistical Groupings ($p < 0.01$)*		
Control	(0 µg/ml)	3.82 ± 1.07	A		
NaHA	(50 µg/ml)	3.92 ± 0.89	A		
NaHA	(1000 µg/ml)	4.48 ± 0.82	A	B	
PSGAG	(1000 µg/ml)	5.00 ± 0.78			C

Common letters denote that groups were not significantly different from each other

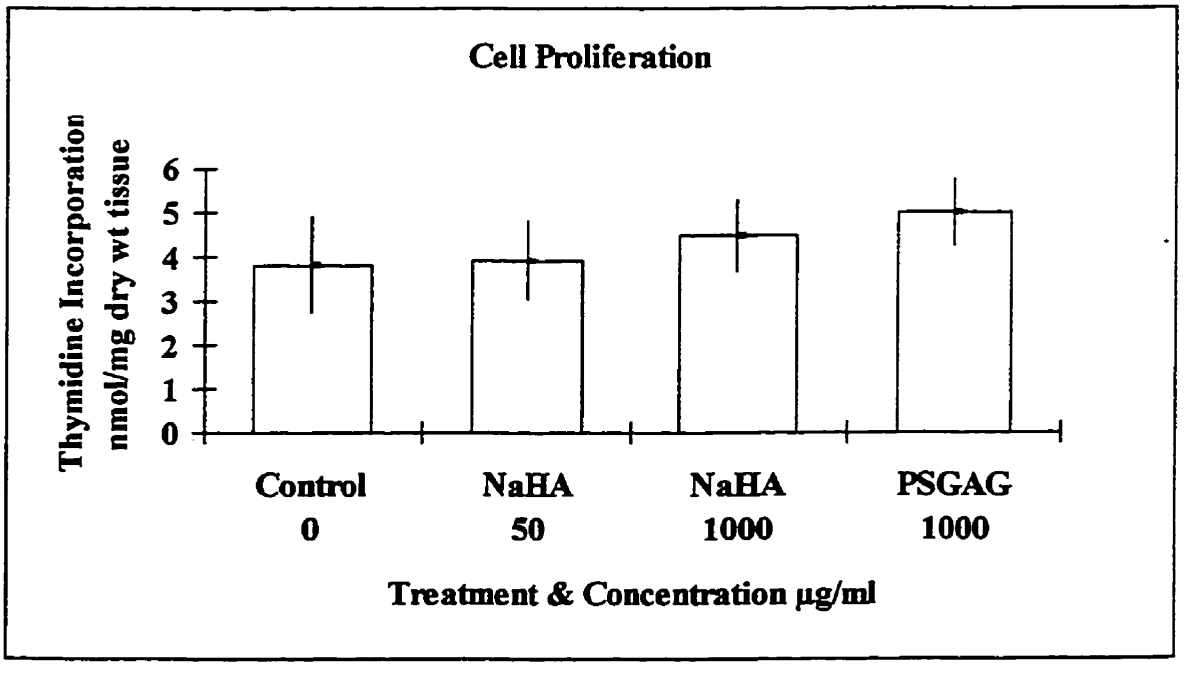

Figure 8.6: Cell Proliferation - Mean thymidine incorporation rate per mg dry weight of equine SDFT by treatment in the culture medium (mean + sd nmol/mg dry weight of tendon)

8.4.7 Tendon Explant Dry Weight

There were significant differences in mean equine SDFT explant dry weights among the control, PSGAG and 1000 µg/ml NaHA treatment groups (table 8.8, figure 8.7). The differences were controlled for in the statistical comparisons above, and did not alter the results of statistical comparisons except for cell proliferation.

Table 8.8: Tendon Explant Dry Weight - Mean explant dry weight by treatment group.

Treatment Group		Explant Dry Wt Mean ± SD mg	Statistical Grouping ($p < 0.05$)*	
Control	(0 µg/ml)	21.06 ± 4.80	A	
NaHA	(50 µg/ml)	19.31 ± 4.79	A	B
NaHA	(1000 µg/ml)	18.29 ± 4.13		B
PSGAG	(1000 µg/ml)	18.51 ± 4.41		B

*Common letters denote that groups were not significantly different from each other.

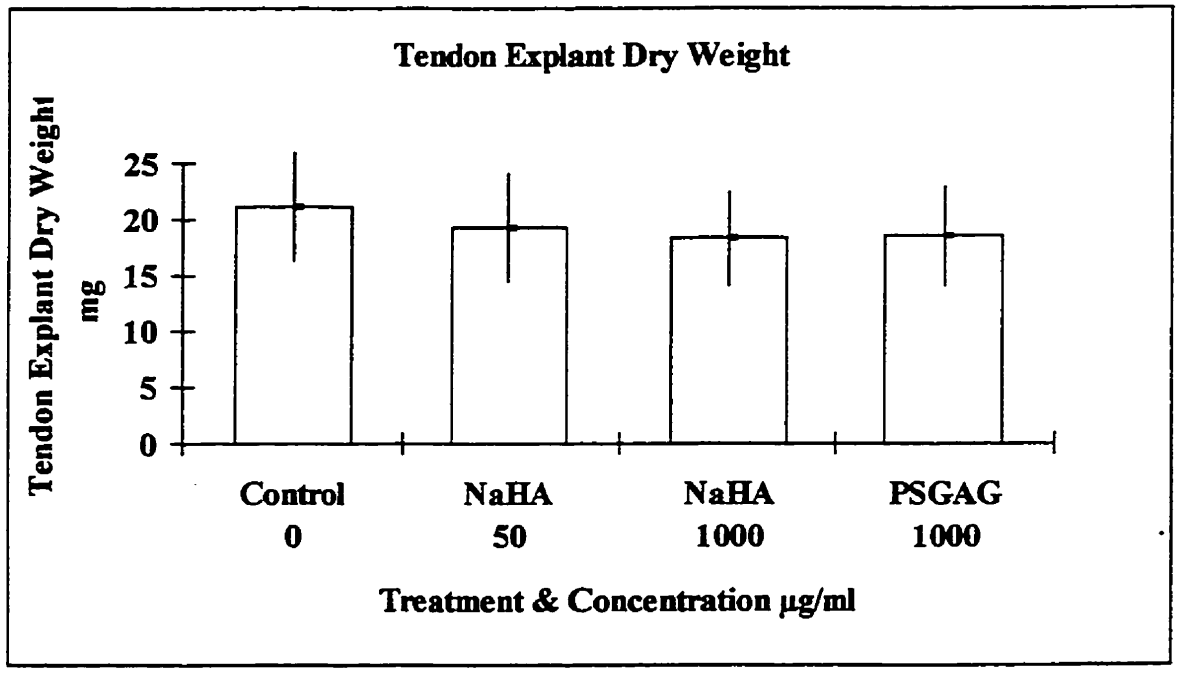

Figure 8.7: Tendon Explant Dry Weight - Mean equine SDFT explant dry weight by treatment group (mean + sd mg dry weight of tendon).

8.5 Discussion

In contrast to experiments four and five, explant dry weights differed among groups by ~ 10%. The power of this experiment was greater than the former two, and therefore may have permitted detection of this difference. The reduction in dry weight may be indicative of general metabolic stimulation - both anabolic and catabolic but this remains to be confirmed. However when this effect was controlled for, the outcome of comparisons of the other variables, except cell proliferation (NaHA group) was not affected. Cultures submitted for mycoplasma evaluation were mycoplasma free.

Increases in rates of total proline incorporation into protein for all treatment groups confirmed the results obtained in experiments four and five but the response to PSGAG was more marked. Although collagen synthesis was increased for the PSGAG and NaHA groups, only PSGAG treatment resulted in greater noncollagen protein synthesis. Therefore it appears that PSGAG is more potent in stimulating protein synthesis than NaHA when compared on a weight basis.

Sulfated GAG synthesis was most marked in response to PSGAG followed by 1000 µg/ml NaHA. However HA synthesis (nonsulfated GAG) was significantly decreased in the PSGAG group. This latter result suggests that the effect of PSGAG is selective with respect to the its effects on GAG metabolism.

8.6 Conclusions

1) Protein, sulfated GAG synthetic rates and cell proliferation in explant cultures of the equine SDFT were significantly increased by exogenous PSGAG or NaHA.

2) The degree of responses to 1000 μg/ml PSGAG was more marked than that of cultures exposed to 1000 μg/ml NaHA.

3) In contrast to sulfated GAG synthesis, HA synthesis was markedly decreased in the presence of PSGAG.

4) A significant decrease in explant dry weight occurred in response to exogenous PSGAG and NaHA, suggesting a possible catabolic effect of these drugs.

5) NaHA at 50 μg/ml does not result in inhibition of protein, sulfated GAG synthesis or cell proliferation.

CHAPTER NINE

9.0 GENERAL DISCUSSION

9.1 General Consideration of the Model

The basic tool utilized for determination of the metabolic responses of the equine SDFT to pharmacologic agents was a modification of a previously developed explant tissue culture system (Riley 1994; Riley *et al* 1996). Representative *in vitro* tissue culture models have been highly valued as devices for studying the biological or biochemical responses of tendon at the cellular and molecular level to cytokines and drugs during the repair phase of tendon healing (Graham *et al* 1981; Gelberman *et al* 1984; Manske *et al* 1984; Mass and Tuel 1991; Russell and Manske 1991; Dahlgren *et al* 1997; Murphy and Nixon 1997). Explant models have been favoured by many workers because they preserve and permit the study of cell-matrix interactions which are considered an essential part of the homeostatic mechanism and repair process in connective tissues (Parry *et al* 1982; Vogel *et al* 1984; Scott 1988; Scott 1990; Abrahamsson 1991; Mass and Tuel 1991; Russell and Manske 1991). This approach has been supported in recent literature as a suitable technique for investigating the effects of pharmaceutical agents and growth factors on the equine SDFT, whereas other workers have employed simple cell cultures for such studies (Riley *et al* 1996; Dahlgren *et al* 1996; Dahlgren *et al* 1997; Murphy and Nixon 1997). The use of RPMI 1640 and 10% DHS in this study was based on extensive comparisons of different sera and media which found this combination to permit satisfactory detection of newly synhesized ECM molecules with reduced variance of results compared to fetal sera or other media (Riley *et al* 1996). It was also felt that this model that was more representative of the *in vivo* repair phase of tendon healing than models employing fetal serum (Riley *et al* 1996; Dahlgren *et*

al 1996; Dahlgren *et al* 1997; Murphy and Nixon 1997). The major limitations of the SDFT explant model used in this study are that, although tendon 'healing' *in vitro* has many similarities with healing *in vivo*, it proceeds at a slower rate, does not permit study of the effect of systemic homeostatic mechanisms on tendon repair, and limits the study of cell-cell interactions, other than those between the tenocytes themselves (Gelberman *et al* 1984; Manske *et al* 1984). To overcome the slower rate of progression the cultures were maintained long-term, recognizing that the effects observed may differ from those observed in short-term cultures (Abrahamsson *et al* 1991a,b). Despite these limitations, it was possible to determine changes in the rates of protein synthesis (collagen and noncollagen protein), polysulfated GAG synthesis, HA synthesis and cell proliferation of tissue from the equine SDFT in responses to exogenous PSGAG, NaHA and rhIGF-I.

9.2 Experimental Animals

Ideally animals should be single sourced and of the same sex, breed, age and strain to eliminate inter-animal variation as a confounding variable. In the chick, rat, cow and to a lesser degree the horse, differences in the histologic and biochemical composition of tendons with age have been demonstrated (Scott 1980; Vogel and Evanko 1987; Scott and Hughes 1986; Vogel and Heinegard 1985; Scott 1988; McCallion and Ferguson 1996; Smith and Webbon 1996). Although it is possible to study identically aged litter mates in the rat, mouse and rabbit studies, it is difficult to attain such rigorous standards in canine studies, and not currently possible in equine studies (Abrahamsson *et al* 1991a, b; Abrahamsson *et al* 1994; Riley *et al* 1996). The age of the animal has also been demonstrated to be an important determinant of *in vitro* responses, with greater rates of biosynthesis and cell proliferation generally attained using fetal or immature tissues (Freshney 1987). An attempt was made in the current study to obtain tendons from young horses approaching skeletal maturity so that the biosynthetic potential of younger animals could be used and the requirement for *in vivo* approximate representation of tendons in which disease occurs could be maintained. The six horses used for the entire study ranged from 14 months to 3.5 years

in age (mean 2.3 years; median 2 years). There were three of each sex, and two Quarter horses, two Quarter horse crosses, a Thoroughbred cross and a purebred Thoroughbred horse. When the horse that was used for development of protocols in experiment one is excluded, the range of ages for comparisons of responses to different pharmacologic agents was from 2 years to 3.5 years (mean 2.5 years; median 2 years). Previous studies by Riley *et al* and Bailey *et al* have demonstrated significant variation between horses with respect to biochemical composition and biologic responses *in vitro*, even when horses were closely matched for breed and age (Riley *et al* 1995; Bailey *et al* 1996; Riley *et al* 1996). Despite these limitations, an effort was made to obtain young untrained, and SDFT injury free horses of similar type and size. However, as observed in the previous studies, significant differences occurred in control values for proline, sulfate and thymidine incorporation between horses. This made confirmation of metabolic changes in response to PSGAG or NaHA necessary by examining cultures from more than one horse. Experiment six confirmed most of the observations made in experiments four and five.

9.3 Discussion of Culture and Analytical Techniques

9.3.1 Equine SDFT Explant Cultures

Some of the variation in the current model was thought to be due to variation in explant size. This problem which was reduced by the use of a standard-sized biopsy punch and the complete exclusion of the epitendon or peri-tendinous tissue in these experiments. There was some variation in explant dry weight between horses. However, within horses at a given time period there were no significant differences detected between groups, except in experiment six (which had 50 explants per group). Standard deviations for explant dry weights were smaller than those attained in previous work, and variances equivalent in the definitive experiments (Riley 1994). The decline in weight over time in experiment three suggested accelerated catabolism either by enzymes of tendinous origin, or exogenous origin. Since this effect had not been observed in the earlier experiments in the present or previous

study and there was no evidence of fungal or bacterial infection, infection with mycoplasma was considered the most likely cause (Freshney 1987; Riley 1994; A. Richardson - personal communication). Mycoplasma testing was incorporated as part of the protocol of experiments four, five and six. The small but significant differences observed in explant dry weight in experiment six may have been a response to treatment with either PSGAG or NaHA, suggesting that these drugs may exert both anabolic and catabolic effects. Changes in dry weight were not observed in experiments four and five. However, sample sizes of at least 50 and 500 respectively, (based on sample size calculations), would have been required to identify the presence any differences that may have been present. In future experiments where there is sufficient tissue, a number of representative explants may be collected following harvest from the horse for dry weight determination and compared to those explants weighed at the end of the culture period. Unfortunately there were too few explants available for this purpose due to the desire to maximize the number of cultures per horse and minimize the number of animals used for the study.

The size of the explants was smaller than those prepared for the previous study so the volume of medium was reduced accordingly to reduce the costs of each experiment (Riley 1994). There was no evidence of necrosis or cellular death as a consequence of these changes based on haematoxylin and eosin or BrdU stained sections of explants (experiment one), and cells were proliferating in these sections at the time of harvest. Later experiments confirmed that cells were synthesizing DNA proliferating, determined by incorporation of radiolabeled thymidine. Although antimicrobial agents were not used after the preparation of the cultures, rates of bacterial or fungal contamination were minimal (1 to 2 explants) to nil when cultures were maintained for up to four weeks.

9.3.2 Radiolabeling and Determination of Radioactive Isotope Incorporation Rates

It has been established that the equine SDFT is metabolically active, but the rates of synthesis of ECM components are low in the mature horse (Birch 1993; Riley 1994). Therefore, assays which are particularly sensitive and have a reasonable degree of specificity

are necessary for evaluation of synthetic responses in this tissue. In previous work using this equine SDFT model, colorimetric methods for the measurement of protein in the culture medium were not sensitive enough, and did not differentiate between newly synthesized protein and protein being lost or diffusing from the explants (Watanabe *et al* 1986; Freshney 1987; Boyer 1991; Riley 1994). Radio labelling of explants and cell cultures with ^{35}S-sulfate, ^{3}H- or ^{14}C-proline and ^{3}H- or ^{14}C-thymidine have been widely accepted as tools for the measurement of PGs (GAGs) synthesis, collagen synthesis and cell proliferation respectively in tendon explant culture studies (Manske *et al* 1978a; Manske and Lesker 1982; Quinones *et al* 1986; Abrahamsson *et al* 1989a,b; Abrahamsson *et al* 1994; Dahlgren *et al* 1996; Riley *et al* 1996; Murphy and Nixon 1997). After incubation with the isotopes, chase incubation with isotope-free medium is recommended to ensure movement of incorporated ^{35}S-sulfate and ^{3}H-proline from the intracellular pool to the extracellular pool and was performed in the current study (Vogel and Peterson 1981; Abrahamsson *et al* 1989b). Radio isotopes which were not incorporated were removed from the medium by size exclusion chromatography (*mw* cut-off of 6000). Therefore unbound isotopes and those incorporated into short peptide sequences or short GAG chains not bound to a protein core were excluded from the measurement of incorporation rates.

Several workers have reported using ^{35}S-sulfate incorporation in order to determine rates of PG synthesis (Abrahamsson 1991; Abrahamsson 1994; Dahlgren *et al* 1996 Dahlgren *et al* 1997). There are several possible sources of error in this assumption, and little attention has been devoted to these limitations in the literature. The first is that the number of sulfated GAG chains per PG molecule varies from one or two per protein core for decorin and biglycan respectively and up to 100 for aggrecan (Jackson *et al* 1991; Kresse *et al* 1993; Gallo and Bernfield 1996). Therefore, there may be a poor correlation between ^{35}S-sulfate incorporation and PG concentration unless synthesis of one PG species predominates. Ideally the PGs should be isolated, the types of PG present identified, and the number of GAG side chains per molecule determined. However, due to the difficulties associated with isolating these highly anionic molecules relatively few have been characterized (Jackson *et al* 1991). Therefore, the level of ^{35}S-sulfate incorporation most accurately reflects rates of sulfated

GAG synthesis rather than PG *per se*. In the SDFT the predominant GAGs are the galactosaminoglycans CS and DS, with lesser amounts of KS (Birch 1993; Bailey *et al* unpublished data). Because CS and DS have three sulfated esters per disaccharide, and KS has one to two sulfated esters per disaccharide, ^{35}S-sulfate incorporation is better correlated with sulfated GAG synthesis than PG synthesis (Gallo and Bernfield 1996). The types of GAGs produced *in vitro* in this study were not determined and although likely to be similar in type to those isolated *ex vivo*, this cannot be assumed to be so (Bailey *et al* 1996). Protocols for the isolation and identification have been developed for evaluation of GAGs in equine digital flexor tendons and would assist in better defining the relationship between ^{35}S-sulfate incorporation and sulfated GAG synthesis in future experiments (Bailey *et al* 1996). Finally, it should be remembered that type III collagen may be cross-linked by disulfide bridges, and it is possible that a small proportion of the ^{35}S-sulfate may be incorporated into such links (Birch 1993).

Proline incorporation and the subsequent separation by RP-HPLC of Hyp and Pro permitted determination of rates of collagen synthesis using a formula based on the ratio of hydroxyproline to proline (Diegelmann and Peterkofsky 1972; Bailey and Light 1989; Abrahamsson *et al* 1994; Murphy and Nixon 1997). Although many authors use this technique, few have typed the collagen to determine the correct ratio of Hyp to Pro for the calculations (table 2.2). *In vivo* studies of equine SDFT healing found that type III collagen predominates during the early repair phase, whereas in a recent *in vitro* study, type I collagen was found to predominate (Williams *et al* 1984; Watkins *et al* 1985a,b; Murphy and Nixon 1997). Collagen was not typed in the present study and the calculations and reported figures for collagen and noncollagen protein were based on the assumption that the collagen produced was predominantly type III (Watkins *et al* 1985a,b). The calculations were also performed based on 30% type III and 70% type I collagen and although absolute values for collagen and noncollagen protein were greater, there were no significant differences in the outcome of statistical comparisons (Williams *et al* 1984). Although proline incorporation was representative of collagen synthetic rates, only those noncollagen proteins containing proline were measured in these experiments. Labeling with radioactive leucine

may be a more accurate measure of total protein synthesis. However, salt precipitation or alternative chromatographic techniques would be required to separate collagen from the noncollagen proteins (Bailey and Light 1989). Given the low rates of synthesis previously identified in this model, this approach may not have been sufficiently sensitive for determination of rates of collagen synthesis (Riley 1994). It is possible that other noncollagen proteins were affected in this study which did not contain significant numbers of proline residues. Proline may also be incorporated into elastin but its synthesis by equine tendon *in vitro* has not been confirmed and less than 1.5% of residues are Hyp.

Thymidine incorporation was used as a measure of cell proliferation and therefore tissue growth. Tissue growth *in vitro* may be due to an increase in cell number, an increase in cell size or increasing the amount of ECM; the latter is usually a variation of the former two parameters (Baserga 1989). Increase in cell number is the most important component of growth in cultures and may be measured directly by counting the number of cells in the cultures, or indirectly by measuring the amount of DNA, RNA, protein or mitosis. Cell counting in the SDFT explants is hampered by the low cellularity of the tissue and the low yield and cell survival from tendon digests (Birch 1993; Bailey *et al* unpublished data). Histomorphometric analysis of 5 μm sections of SDFT explants is a difficult and time consuming technique (Riley *et al* 1996). The amount of DNA per cell is usually constant and its measurement is the method of choice for evaluating growth and cell survival in solid tissue cultures and its measurement may be easier than counting cells in the SDFT explants (Baserga 1989; Riley 1994). This technique was not used in the equine SDFT cultures due to the problem of low cell density mentioned. Counting of mitotic figures either directly or with the assistance of a stain such as BrdU is an indication of cell proliferation but not growth (Baserga 1989). Thymidine incorporation is also limited to estimating the amount of cell proliferation and does not permit the study of the quiescent cell population. However it is a sensitive and powerful assay which is widely used to quantitat cell proliferation (Baserga 1989). Additionally, it may be employed in autoradiographic procedures in a qualitative manner (as can the BrdU assay) in order to determine which cell types are undergoing mitosis (Baserga 1989; Matsuda 1994). In the current study, ^{14}C-thymidine was

found to be an effective tool for quantitative comparisons of the effects of NaHA, PSGAG and rhIGF-I.

9.3.3 Pre-column Dabsyl Chloride Derivitization and Reverse Phase High Performance Liquid Chromatography

Dabsyl amino acids are highly chromophoric compounds produced by the reaction of DABS-Cl with amino acids in alkaline conditions (Lin 1984). The technique developed and used in this study enabled separation of Hyp and Pro in nanomolar amounts and the subsequent measurement of radioactivity of fractions corresponding to each peak is permitted the calculation of rates of incorporation into collagen and non-collagen protein (Lin 1984; Drnevich and Vary 1993; Ikeda *et al* 1993). Dabsyl chloride was used as a derivatization agent because the dabsyl amino acids are stable for several months at -20^{0}C, the derivatization time with DABS-Cl is short compared to other agents, and it reacts with both primary and secondary amino groups (Ikeda *et al* 1993). *O*-phtalaldehyde reacts with only primary amino groups and did not improve detection of Hyp and Pro in this study (Ikeda *et al* 1993). Initially digests of the explants were derivatized using a published protocol, separated by RP-HPLC and absorbencies measured at 425 nm (Lin 1984; Drnevich and Vary 1993). However the relatively particulate nature of the tendon digest after filtering resulted in rapid clogging of the pre-column filter and most of the Hyp and Pro separated was associated with collagen that was present in the explant at the time of harvest from the horse. The high background concentrations resulted in markedly reduced SPA and the peak resolution and separation were poor. Therefore newly synthesized protein was precipitated from the medium, dried, digested, derivatized and separated. The absorbance detector was exchanged for a fluorescence detector set at 436 nm. Detection in the visible range at 436 nm decreases the interference caused by UV absorbing material in biological samples (Ikeda *et al* 1993). The sensitivity of the dabsylation RP-HPLC technique is enhanced by 5 to 10 times when a fluorescence detector is used instead of a photometer based absorbence detector (Lin 1984; Hancock and Harding 1984). This resulted in chromatographs with

peaks that were smaller with excellent resolution and separation that was sensitive enough to discriminate between the 3-OH and 4-OH isomers of Hyp (Bailey and Light 1989). The derivatization procedure was particularly pH sensitive and great care had to be taken to maintain conditions at pH = 9.0 for successful dabsylation and separation (Drnevich and Vary 1993).

The formulation of the two elution solvents and the program used was initially based on the work of Drnevich and Vary (1993). Success using this protocol was limited with the available equipment, so an alternative protocol was developed based on two further publications (Negro *et al* 1987; Ikeda *et al* 1993). Flow rate was decreased to conserve solvents and the column temperature and percentage mix of solvents were arbitrarily adjusted until satisfactory resolution and minimum development time were obtained. Maintaining stable conditions was difficult because much of the equipment required maintenance and repair. However once these problems were resolved 20 µl samples could be separated every 30 minutes and fractions collected for scintillation counting. Ideally an in-line radioactivity counter would permit more rapid collection of data and disposal of radioactive wastes (Macek *et al* 1989).

9.4 Pilot Studies

Following the establishment of the equine SDFT explant tissue culture system and the suitable analytical procedures, two pilot studies were conducted. In previous tendon explant culture studies it was found that rates of cell proliferation and synthesis of macromolecules differed depending upon the duration of the culture period (Abrahamsson *et al* 1989b; Abrahamsson *et al* 1991b; Riley 1994). When equine SDFT explants were cultured for up to four weeks in RPMI 1640 the variances of isotope incorporation rates were higher at week two than at week four (Riley 1994). Because the mechanism of action of PSGAG and NaHA as pharmacologic agents on tendons has not been elucidated it was not known what dose or duration of treatment was required in order to initiate a response, if any. The recommendation for treatment of articular disease in the horse with PSGAG is

to administer a dose every four days for eight injections (Dow *et al* 1996; Luitpold Pharmaceuticals, Animal Health Division, Shirley, NY, USA), whereas NaHA is given weekly for three treatments (Swanstrom 1978). The pilot studies were designed to give some indication of the optimal pre-treatment stabilization period which would minimize variability during the testing of the drugs and of the duration of treatment required for a measurable response. In experiment two, three of the groups that were cultured for a total of 21 days prior to radiolabeling were exposed to treatment for three days and four were exposed to six days of treatment. This approach differs from that of other long-term *in vitro* tendon explant studies in which the explants were continuously exposed to the agent of interest throughout the culture period (Abrahamsson *et al* 1991b; Murphy and Nixon 1997). It is more likely that *in vivo* administration with either PSGAG or NaHA would occur at intervals of four days or one week respectively according to current recommendations for these drugs (Marr *et al* 1993). For this reason, and the apparent greater reproducibility of the 6-day treatment protocol, we elected to conduct all subsequent experiments with a 6-day treatment period.

In horses affected with tendinitis, subclinical injury is usually present before the onset of a more severe acute episode of tendinitis (Stromberg and Tufvesson 1969; Silver *et al* 1983; Genovese *et al* 1985; Dow *et al* 1991; Marr *et al* 1993). Therefore the treatment of most injured horses occurs at some time after injury during the phase of healing predominated by reparative responses (Dow *et al* 1991; Marr *et al* 1993). After injury, *in vivo* inflammation predominates in the first few days, mediated by neutrophils and macrophages which stimulate both cell death and activation of previously quiescent mesenchymal cells (Chvapil 1996). Similarly, in explant cultures some cells die, some remain quiescent and some proliferate, differentiate and migrate (Freshney 1987). However this process is neither initiated nor controlled systemically or locally by mediators produced by inflammatory cells. Rather, its course is determined by the degree of tissue damage caused during establishment of the cultures and by the environmental and nutrient media conditions of the culture system. Although tendon healing *in vitro* has many similarities with healing *in vivo*, it proceeds at a slower rate without systemic regulation (Gelberman *et al* 1984; Manske *et al* 1984). *In vivo* the maximal rate of cell proliferation occurs by day seven, and of

collagen synthesis by day ten (Chvapil 1996; Davis 1996). From the results of experiments two and three it is evident that protein and GAG synthesis were still increasing between 15 and 21 days of culture, and possibly after day 21. More data points are necessary to describe the relationship between time and synthetic rates *in vitro* and determine the time of peak synthesis but it is apparent that it is delayed compared to that reported for *in vivo* healing (Gelberman *et al* 1984; Manske *et al* 1984; Abrahamsson 1991). It is evident from previous work with the equine SDFT *in vitro* that differences in synthetic rates occur with time and interestingly the response to some of the treatments in the present study differed according to the duration of the pre-treatment culture period (Riley 1994). In experiment two after a nine-day pre-treatment culture period NaHA and PSGAG appeared to inhibit protein and GAG synthesis but after a 15-day pre-treatment culture period there was no significant difference for the NaHA group and increased synthesis in the PSGAG group. These differences will be discussed further in the sections addressing the responses to each drug. Following experiments used a compromise pre-treatment culture period of 18 days and concentrated on increasing sample size to improve the statistical power of each trial.

A treatment group containing rhIGF-I was included as a positive control group in both pilot studies. Studies using explant cultures of rabbit flexor tendons indicate that IGF-I has a potent anabolic effect on protein synthesis and cell proliferation in serum-free and supplemented media (Abrahamsson *et al* 1991a,b). It was thought that rhIGF-I would be useful as a positive control in the equine SDFT model when evaluating the effects of NaHA and PSGAG. Groups combining each drug and rhIGF-I were also included to determine whether interactive effects were possible. The doses of drugs used in the pilots were based on other publications which had investigated them in other species or other connective tissues (Abrahamsson 1991; Nethery *et al* 1992; Salti *et al* 1993). The response to rhIGF-I was poor in both experiments (even though rhIGF-I from two different sources was used) so it was considered unsuitable as a positive control group at the dose tested. Therefore, dose response studies were conducted in experiments four and five to further evaluate the response of the equine SDFT explants to rhIGF-I.

9.5 Polysulfated Glycosaminoglycan (Adequan®)

The positive anabolic effects of exogenous PSGAG on cartilage have been reported previously but this study is the first to confirm comparable effects on the equine SDFT (Adam 1982; von der Mark 1982; Glade 1990; Ghosh *et al* 1992; Steinmeyer *et al* 1992). These effects were first observed in experiment two and examined more critically and confirmed in experiments four and six. Interpretation of the results of experiment three were confounded by a decline in explant size suspected to be due to contamination with mycoplasma. After experiment three all subsequent experiments were tested and found to be negative for mycoplasma. Prior to the initiation of this study, the effects of PSGAG on the equine SDFT had only been investigated under clinical conditions following administration of either intra-lesional, peri-lesional or systemic Adequan® for tendinitis (Redding *et al* 1992; Goodship *et al* 1992; Marr *et al* 1993; Barr *et al* 1995; Dow *et al* 1996). However, in the only one of these studies with a control group, there were no significant differences between the percentage of Thoroughbred horses returning to work following a course of intratendinous or intramuscular PSGAG and those returning to work following conservative management alone (Marr *et al* 1993).

In the horse only one *in vitro* study investigating the effect of PSGAG on isolated tenocytes has been published (Dahlgren *et al* 1996). This study utilized a model based on equine tendon fibroblasts in monolayer culture and examined the responses to doses of PSGAG ranging from 25 to 200 μg/ml (Dahlgren *et al* 1996; Dahlgren *et al* 1997). It was concluded from this study that neither proteoglycan synthesis nor cell viability were affected compared to controls. The equine SDFT explant model was chosen in the current study in order to maintain cell-matrix interactions. The effects of PSGAG were examined over the dose range 100 to 5000 μg/ml and a dose dependent increase in the rate of sulfated GAG synthesis was found. This compares to an approximate CS concentration of 2.5 μg/mg wet weight in normal equine SDFT of horses of similar age estimated on the basis of data obtained in biochemical analyses (Birch 1993). The results concur with those of Dahlgren *et al* in that neither protein (collagen and noncollagen) nor sulfated GAG synthesis were

significantly different from the control group at concentrations less than 500 μg/ml (Dahlgren *et al* 1996). However, at concentrations greater than or equal to 500 μg/ml there were significant increases in the rates of protein synthesis (collagen and noncollagen protein) and sulfated GAG synthesis, and it appeared that the degree of response was dependent upon the concentration of PSGAG in the culture medium. In short term studies of equine explant cultures of articular cartilage, exogenous PSGAG at concentrations of 12500, 25000 and 50000 μg/ml on collagen and sulfated GAG synthesis were examined and the latter two concentrations were found to increase ^{35}S incorporation by 120 and 316% respectively (Glade 1990). The latter two concentrations are similar to those present in synovial fluid after intra-articular injection of 250 mg PSGAG but the reported concentration after intra-muscular injection of 500 mg is only 0.25 μg/ml in normal horses (Glade 1990; Burba *et al* 1993). In a later study, six days of exposure to PSGAG at 200 μg/ml was found to inhibit PG (GAG) synthesis and had no effect on PG breakdown in short term equine explant cultures of articular cartilage (Caron *et al* 1991). When a similar study was performed with cartilage from osteoarthritic middle carpal joints at a concentrations of 25 μg/ml and 25000 μg/ml similar findings were made (Caron *et al* 1993). The reasons for the contrary finding in the studies by Caron and workers is unknown, but ^{35}S-$NaSO_4$ was added at the same time as PSGAG and there is no mention in the experimental materials and methods of the SPA of the ^{35}S or whether or not this was corrected for the concentration of sulfate due to PSGAG in the medium. In the current study, all rates of synthesis were based on the corrected SPA for each isotope which was determined by considering the concentrations of radioactive and non-radioactive of proline and sodium sulfate in the culture medium (Riley *et al* 1996). Culture medium RPMI 1640 is thymidine free.

The significant increase in sulfated GAG and noncollagen protein strongly suggests that PG synthesis was increased. However, confirmation of this awaits polyacrylamide gel electrophoresis of protein precipitated from the medium and staining with dimethylmethylene blue (DMMB) or specific antibodies. Chromatographic separation and digestion of GAG sidechains would be required to confirm this supposition (Caron *et al* 1993; Bailey *et al* 1996). Increased amounts and molecular weights of PG are synthesized in a dose dependent

manner by cultured human and rabbit articular chondrocytes in response to exogenous PSGAG, but not in chick embryo or rabbit meniscus fibroblast cultures (Burkhardt and Ghosh 1987; Ghosh *et al* 1992). In cultured osteoarthritic human and chick cartilage PSGAG induced significant increases in PG (the core protein fraction and GAGs) synthesis, particularly in PG fractions associated with link protein (Adam 1982). Investigations into the distribution and binding of PSGAG have concluded that the drug has a greater affinity for PG and noncollagen proteins than for collagen (Ghosh *et al* 1992). The present study did not specifically investigate the binding affinities of PSGAG for different macromolecules in the SDFT-ECM, but there were no great differences among the relative degree or rates of collagen, noncollagen protein or GAG synthesis. It is possible that the type of PG produced in the treatment groups differed from that produced by the control group. In normal tensile SDFT the predominant PGs are decorin, biglycan and fibromodulin and in the metacarpophalangeal regions levels of CS compatible with the presence of aggrecan in the metacarpophalangeal regions have been detected (Smith and Webbon 1996). However, these results conflict with studies that have determined that CS (the major GAG in versican and aggrecan) is the predominant GAG followed by DS (the major GAG in decorin and biglycan) in the tensile SDFT (Birch 1993; Bailey *et al* 1996; Gallo and Bernfield 1996). Because the SDFT explants were not under tension or compression it is possible that the types PG synthesized *in vitro* may differ from those normally synthesized *in vivo* in response to motion and tension (Koob and Vogel 1987b; Okuda *et al* 1987; Kubota *et al* 1996; Nabeshima *et al* 1996). Since the present study has established a significant response to PSGAG it would be of interest in future studies to quantify the different types of GAGs and PGs produced.Very few of the core proteins of PGs have been isolated and their structure characterized and attempts to do so have had limited success because their high charge density makes separation and purification difficult (Jackson *et al* 1991; Caron *et al* 1991; Caron *et al* 1993). In a recent investigation, PSGAG at 10 and 100 μg/ml increased KS synthesis (the predominant GAG of fibromodulin) in cultured canine articular chondrocytes (Steinmeyer *et al* 1992). In the previously mentioned study investigating the effect of PSGAG on equine cartilage from osteoarthritic middle carpal joints, the only significant

anabolic effect identified was an increased concentration of KS substitution on the core protein of an unidentified large PG (Caron *et al* 1993).

Protein synthesis was stimulated in a dose-dependent manner in this study. The results concur with those of the study quoted above which investigated the response of canine articular cartilage chondrocytes to PSGAG and concluded that the response was not specific because both collagen and noncollagen protein synthesis were increased (Steinmeyer *et al* 1992). Collagen synthesis by equine articular cartilage explants also increases in response to PSGAG in a dose dependent manner (Glade 1990). The objectives of the study included determination of the effect of PSGAG on collagen and noncollagen protein. As discussed above, now that an anabolic effect has been observed, the purification, isolation, identification and quantifying individual proteins would be useful in future experiments to more accurately document qualitative changes in the distribution of ECM macromolecules. Of particular interest would be collagen typing since the production of larger fibril diameter type I collagen is considered desirable to optimize scar strength *in vivo* (Williams *et al* 1984; Watkins *et al* 1985b). The present study reports on the metabolic responses of the tissue, particularly the cells, to exogenous PSGAG. In rat tendon, the GAG moieties of the PGs interact electrostatically in a reversible manner with type I collagen with an affinity which is dependent upon molecular shape and linear charge density. This provides up to 25% of the stabilization forces in tendon collagen (Nimni and Harkness 1988; Scott 1990). Naturally occurring CS accelerates fibre formation during the nucleation phase, but when CS or PGs are added after the nucleation phase the growth phase of fibrilogenesis is delayed, limiting fibril diameter (Nimni and Harkness 1988; Parry and Craig 1988). Further *in vitro* and *in vivo* investigation into the interactive effects between collagen and PSGAG would be of value in elucidating the complex responses to CS.

Hyaluronic acid was markedly decreased (46%) in the culture medium of SDFT explants treated with PSGAG at 1000 μg/ml compared to the control group. This result contrasts with reports in which HA synthesis by chondrocytes and synovial cells was markedly increased by PSGAG (up to 250% at 100 μg/ml) and the average molecular weight of HA synthesized was increased (Greiling 1982; Nishikawa *et al* 1985; Smith and

Ghosh 1986; Burkhardt and Ghosh 1987). In synovial membrane *ex vivo* explant cultures, HA synthesis was increased in rabbits after systemic injection of chondroitin-4-sulfate and chondroitin-6-sulfate (Nishikawa *et al* 1985). An explanation of the cause of decreased HA in the medium in experiment eight is speculative. In synovial cells the action of PSGAG appears to result from binding to receptors on the cell surface and it likely that this occurs on the surface of the SDFT tenocytes (Ghosh *et al* 1992). Hyaluronate differs from other GAGs because it is synthesized in the plasma membrane. Although hyaluronidase has been isolated from lung fibroblasts, most HA is not degraded locally but by reticuloendothelial cells in the liver (Sampson *et al* 1992; Gallo and Bernfield 1996). Therefore, the decrease in HA in the medium may have resulted from inhibition of HA synthetase by binding of PSGAG to the plasma membrane and/or by binding and sequestering cytokines that are essential for the stimulation of HA synthesis (Sampson *et al* 1992). Although this effect has not been demonstrated for HMW-HA, a recent study found that a very low molecular weight form of NaHA consisting of hyaluronate hexasaccharide (HA_6) inhibited HA synthesis by blocking receptors and displacing endogenous HA from chondrocytes in culture (Knudsen 1993). Alternatively, PSGAG may up regulate CD44 receptors and enhance binding of synthesized HA to these membranes or HA may become bound in aggregates associated with the increased levels of synthesis of PG and LP, resulting in decreased levels of HA liberated into the medium. In future experiments an isotopic assay for HA may give more information on newly synthesized HA levels associated with the explants themselves (Nishikawa *et al* 1985).

In addition to stimulating the synthesis of macromolecules, exogenous PSGAG at 1000 μg/ml also stimulated cell proliferation in experiment six. *In vitro* studies of synovial cells exposed to exogenous PSGAG have shown antimitotic effects (Trotter 1996). However studies of cartilage have demonstrated stimulated proliferation in response to lower doses of PSGAG (Vacha *et al* 1984; Glade 1990). Thymidine incorporation into equine articular cartilage cultures doubles at 12500 μg/ml PSGAG, is not affected by 25000 μg/ml, and is inhibited by 50000 μg/ml of PSGAG. A dose response study planned to examine the relationship between PSGAG concentration and thymidine incorporation was not completed

due to logistical problems in experiment four but based on the effects upon cartilage should be considered for future investigation.

In experiment, two after a nine-day stabilization period PSGAG appeared to inhibit protein and GAG synthesis, but after a 15-day pre-treatment culture period there was increased synthesis in the PSGAG group. However a dose of 2000 μg/ml PSGAG was used in the nine-day pre-treatment period group. The inhibitory response may be due to an interaction of the time of treatment and dose. In experiment four (effect of concentration of PSGAG on explants) it appeared that optimal synthetic response at 1000 μg/ml PSGAG with an apparent decline in response to concentrations >1000 μg/ml PSGAG. Although statistical support for this apparent declining or inhibitory response was lacking, the apparent trend warrants further investigation of the possible toxic effects of PSGAG at concentrations higher than 1000 μg/ml PSGAG. Other studies have shown inhibition of GAG synthesis by synovial cells exposed to 300 mg/ml (Kleesiek and Greiling 1982). Thus, it would be of value to more closely examine the effect of PSGAG on different phases of the healing process *in vivo* and *in vitro*.

Many of the effects of PSGAG have been identified *in vivo* and *in vitro*, but the precise mechanism of its action upon cells is unknown (Trotter 1996). Presumably the response to PSGAG is mediated by interactions with cell surface associated proteoglycans and/or integrins. There is evidence that such interactions are dependent upon the degree of sulfation of the disaccharides. For example, when HA is synthetically polysulfated its biological activity is markedly enhanced (Chang *et al* 1994). The principal GAG present in PSGAG is CS which is highly anionic due to an average of three to four sulfate esters per disaccharide thus endowing PSGAG with the capacity to covalently bind many molecules (Ghosh *et al* 1992). Because its affinity for PG and noncollagen protein is reportedly greater than that for collagen it is possible that PSGAG binds to the cell surface associated proteoglycans to exert its effect (Andrews *et al* 1985). There is little specific information on the interactions between PSGAG or other sulfated GAGs and cell surface receptors or on the transmission of signals to the intracellular space (Labat-Robert *et al* 1990; Ciarelli *et al* 1996).

Although the PSGAG appeared to increase rates of synthesis of collagen, noncollagen protein and GAGs, it is possible that this effect was in part or totally the result of decreased catabolism (Ghosh *et al* 1992). The reported effects on catabolism are the most widely known of PSGAG properties. It is a competitive inhibitor of neutrophil elastase and an inhibitor of cathepsin B_1 and a strong inhibitor of ß-glucuronidase, ß-galactosidase, ß-*N*-acetylglucosaminidase, α-glucosidase, α-mannosidase, KS glycanohydrolase, chondroitin sulfotransferase, neutral protease, ß-glucuronidase, myeloperoxidase, cathepsin G, and human granulocyte elastase (Baici and Fehr 1982; Trnavsky 1982; Ghosh *et al* 1992). In an *in vivo* lapine cartilage model, significant suppression of MMPs and serine proteinase activity occurred after intra-articular administration of PSGAG (Howell *et al* 1986). In enzyme assays, its effect upon MMP synthesis appears to be dose and species dependent (Nethery *et al* 1992). Rat collagenase activity is increased by PSGAG concentrations > 0.5 µg/ml up to a dose of 50 µg/ml, and human collagenase activity increases at concentrations > 5 µg/ml (Nethery 1992). In the same study, stromeolysin from both species was inhibited in a dose dependent manner over the concentration range 0.5 to 5000 µg/ml PSGAG. Samples were collected during the present study for detection and quantifying collagenase, stromeolysin and TIMPs in order to determine if these molecules played a role in the observed synthetic responses. Stromeolysin and collagenase activity were identified in these samples, but data for comparative analysis are not yet available.

9.6 Sodium Hyaluronate (Hyonate®)

The results of this study indicated that exogenous NaHA exerts positive anabolic effects on equine SDFT explants in a dose related manner. There is little comparative information on the effects of NaHA on soft connective tissues. It inhibits granulation tissue formation *in vivo*, possibly by inhibiting the chemoattraction of fibroblasts by macrophages (Rydell 1970; Rydell and Balazs 1971; Balazs 1985). However in the current study macrophages were excluded from the equine SDFT explant model. Studies in rabbits with traumatized digital tendon sheaths treated with NaHA showed less connective tissue reaction

and scarring, or no significant improvement in wound healing (Rydell and Balazs 1971; Thomas *et al* 1986). Little is known of its mechanism of action in joints or tendons because studies reported in the horse have been clinical trials in which few or no biochemical parameters have been measured (Churchill 1985; Nixon and Gaughan 1989; Spurlock *et al* 1989b; Gaughan *et al* 1991; Gift *et al* 1992). The current study is the first to determine the metabolic responses of the equine SDFT to NaHA.

The effects of NaHA were examined over the dose range 50 to 2000 μg/ml. The upper concentration was limited by the relatively low concentration of the available commercial preparation. In experiment five an apparent dose dependent increase in the rate of sulfated GAG synthesis, total protein synthesis and collagen synthesis was found. These were significant only at the 1000 and 2000 μg/ml. *In vitro* studies of NaHA at concentrations < 1.0 μg/ml found inhibition of PG synthesis by chondrocytes but no inhibition occurred in muscle, mature human skin or human synovial fibroblasts at concentrations up to in 10 μg/ml (Ghosh *et al* 1992). In one of the few studies investigating the effects of the drug at a higher concentration, NaHA at 100 μg/ml increased glucosamine incorporation into HA by primate fibroblasts (Balzacs 1985). The concentrations of NaHA examined in the current model were much higher than those examined in other *in vitro* studies and the anabolic effects on macromolecular synthesis occurred only at the upper end of the dose range. This may explain the difficultly that has been experienced establishing its effect on tendon healing *in vivo* because very high concentrations seem to be required at the site of injury in order to observe an effect, and the commercial preparation tested comes only in 20 mg/ml concentration (Gaughan *et al* 1991; Gift *et al* 1992; Gaughan 1995).

In addition to stimulating the synthesis of macromolecules, NaHA at 1000 μg/ml also stimulated cell proliferation in experiment six. In experiment five, cell proliferation appeared to be inhibited by 50 μg/ml NaHA but in experiment six there was no difference among the 50 μg/ml NaHA and control groups. In experiment six there were more explants per treatment group and increased cell proliferation was identified in the 1000 μg/ml NaHA. There was a concurrent decrease in dry weight in experiment six. However, statistical analysis yielded the same results whether of not this was controlled in comparisons.

Although there is a limited understanding of some of the chondroprotective and anti-inflammatory effects of NaHA, the mechanism of action which resulted in increased synthesis and cell proliferation is unknown and previously unreported in the horse (Trotter 1996). Recent work with canine articular cartilage *in vivo* determined that HMW-HA (Hyalovet®) is capable of down-regulating stromeolysin and/or its regulatory pathway through TNF-α (Comer *et al* 1996). Furthermore, when HA is artificially sulfated its effect is increased 5000 fold (Chang *et al* 1994). In studies of synovial cells HA receptors are preferentially stimulated by HMW-HA or inhibited by LMW-HA (Ghosh *et al* 1992). It is possible that the synthetic effects of NaHA are mediated through the same or related HA receptors and that a high concentration of NaHA is required to saturate these receptors and promote a more generalized synthetic response (Gallo and Bernfield 1996).

9.7 Comparison of Polysulfated Glycosaminoglycan and Sodium Hyaluronate

Many of the specific responses observed in experiment six have been discussed above. The degree of response at comparable weight based concentrations (1000 μg/ml) was less marked for cultures treated with NaHA than those treated with PSGAG. This concentration was chosen for comparison based on the apparent optima identified in the dose response studies for PSGAG and NaHA. The molecular weight of the drugs used in the study were not measured for this experiment but have been estimated in other studies to be 10,000 and 321,600 for PSGAG (Adequan®) and NaHA(Hyonate®) respectively (Steinmeyer *et al* 1992; Uden and Lavoie 1997). Although the molar concentration of NaHA was lower than that of the PSGAG, the number of disaccharides was several orders of magnitude greater. These findings agree with those of others that ligand binding with the sulfate groups on PSGAG results in stronger interactions and more pronounced metabolic effects than the much weaker reversible binding that occurs with the carboxylate moieties of the NaHA molecules (Nimni and Harkness 1988; Chang *et al* 1994).

In addition to the significant effects on protein, sulfated GAG synthesis and cell proliferation, there were small but significant decreases in explant dry weight of the 1000

μg/ml NaHA and PSGAG groups. This response was not observed in experiments four and five and explant dry weights differed among groups by ~ 10%. The size of treatment groups in experiment six was greater than the former two studies and therefore may have assisted the detection of this difference. This difference may be associated with an inter-horse effect as identified in previous studies, but it is also possible that the reduction in dry weight may be indicative of general metabolic stimulation - both anabolic and catabolic (Riley 1994; Bailey *et al* unpublished data). Such a dichotomous response has been reported previously when PSGAG was found to stimulate collagenase activity while at the same time inhibiting stromeolysin activity *in vitro* (Nethery *et al* 1992). The present study was designed to investigate the possible anabolic effects of NaHA and PSGAG. Further investigation of the catabolism of the macromolecules of the ECM is recommended in future studies. Despite the reduction in dry weight of the treated cultures, when this effect was controlled for in statistical analysis the outcome of comparisons of the variables (other than cell proliferation for the 1000 μg/ml NaHA group) was not affected. Cultures submitted for evaluation were mycoplasma, bacteria and fungus free.

9.8 Insulin-like Growth Factor I

Insulin-like growth factor I is a basic peptide consisting of 70 amino acids which is synthesized predominantly by the liver and occurs in high concentrations in serum but low concentrations in the tissues (Froesch *et al* 1985; Daughday and Rotwein 1989). Recently the role of rhIGF-I-I in tendon metabolism has been investigated *in vitro* (Abrahamsson *et al* 1991a,b; Lin *et al* 1991). Based on the potent effect of rhIGF-I on rates of matrix synthesis by rabbit tendon flexor tendon, rhIGF-I was selected as a positive control against which the responses of equine SDFT explants could be compared. A concentration of 100 ng/ml was chosen for the pilot studies (experiments two and three) based on previous works and budgetary constraints (Abrahamsson *et al* 1991a,b; Lin *et al* 1991). In experiment two rhIGF-I in combination with PSGAG or NaHA, but not singly, resulted in increased protein and sulfated GAG synthesis in the groups which were treated for six days after a pre-

treatment culture period of 15 days. The magnitude of the response compared to the control group was small. The rhIGF-I product used had not been previously culture tested, and following the poorer than expected response, an alternative source if rhIGF-I was used in experiment three. However, significant anabolic responses with rhIGF-I were not observed in experiment three, possibly due to mycoplasma contamination.

These results led to two rhIGF-I dose response studies in experiments four and five. When the effects of concentrations of rhIGF-I between 50 and 500 ng/ml on SDFT explants from a 3.5 years old horse were compared to a control group, the only significant difference was an increase in collagen synthesis of only 15.6% at 500 ng/ml rhIGF-I. However, when concentrations of rhIGF-I between 5 and 100 ng/ml were evaluated in SDFT explants from a younger horse, mean rates of protein (collagen and noncollagen protein), sulfated GAG synthesis and cell proliferation were significantly elevated by up to 126% (114% and 176%), 128% and 41%, respectively. As well there was a clear dose-response relationship between the concentration of the rhIGF-I in the range of 5 and 10 to 25 ng/ml, depending upon the parameter being measured. The interaction and synergistic effects of rhIGF-I with other cytokines has been well documented (Lin *et al* 1991).

When short term cultures of embryonic chicken tenocytes were cultured in rhIGF-I at 1, 5, 10, 100 and 1000 ng/ml in the presence of 0.5% FBS, DNA synthesis was significantly increased at 5 to 10 ng/ml (Lin *et al* 1991). Short term rabbit flexor tendon explant cultures exposed to 10, 25, 50, 100, 250, 500 and 1000 ng/ml of rhIGF- I in serum free medium increased PG synthesis in a dose dependent manner between 10 and 1000 ng/ml (Abrahamsson *et al* 1991a). Collagen and noncollagen protein synthesis increases between 10 and 500 ng/ml, and 10 and 250 ng/ml rhIGF-I occurred in a dose dependent manner and there was decline in rates of synthesis at higher doses. Stimulation of cell proliferation by rhIGF-I was dose responsive between 10 and 100 ng/ml. In similar long term cultures exposed to rhIGF-I at 50 ng/ml, PG, collagen and noncollagen protein were significantly increased compared to controls (Abrahamsson *et al* 1991b). It is possible that the higher doses of rhIGF-I used in the experiment two were too high and prevented the agent from exerting an effect on synthetic responses. The reason for the lack of effect of rhIGF-I at

higher concentrations is not known and little attention has been given to this observation (Froesch *et al* 1985; Abrahamsson *et al* 1991a; Lin *et al* 1991). In all of these studies young animals were used for the cultures. It is possible that the lack of response to rhIGF-I in experiment two was an animal effect because the horse was almost two times older than that used in experiment three. Thus, even though some of the concentrations of rhIGF-I were similar to those evaluated in experiment five, there were no significant differences in rates of synthesis.

Recently, other workers have evaluated the effects of rhIGF-I at concentrations of 100, 250 and 500 ng/ml on long term explant cultures of the equine SDFT in the presence of 5% FBS (Murphy and Nixon 1996). There were some similar findings to those of experiment four. Only in the 500 ng/ml rhIGF-I treated explant was GAG synthesis increased. However, this was not significantly different from the control group (Murphy and Nixon 1996). Likewise, cell proliferation was not significantly affected by rhIGF-I over the dose range tested. Collagen synthesis, on the other hand, was significantly elevated at all concentrations with an apparent dose dependent relationship. The data from the rhIGF-I trial in experiment four supports this finding, and in addition, confirms the stimulation of sulfated GAGs and cell proliferation observed in rabbit tendon experiments.

The equine cultures of Murphy and Nixon were treated with rhIGF-I in the presence of 5% FBS which has high concentrations of other growth factors including IGF-I (Jayme 1990; Riley 1994; Murphy and Nixon 1996). The present study used homologous species specific serum which is more uniform in composition from batch to batch because the nutrient medium and the responses of the SDFT *in vitro* are more likely to be representative of the *in vivo* response to IGF-I and other drugs (Manske *e al* 1984; Jayme 1990; Riley 1994).

9.8 Conclusions

9.8.1 Polysulfated Glycosaminoglycans (Adequan®)

1) Mean rates of protein synthesis by cultured equine SDFT explants as determined by proline incorporation were significantly increased in the presence of exogenous PSGAG at concentrations between 500 to 5000 μg/ml in a dose-dependent manner.

2) Mean rates of collagen synthesis by cultured equine SDFT explants were significantly increased in the presence of exogenous PSGAG at concentrations between 500 to 5000 μg/ml in a dose-dependent manner.

3) Mean rates of noncollagen protein synthesis by cultured equine SDFT explants were significantly increased in the presence of exogenous PSGAG at concentrations between 100 to 5000 μg/ml in a dose-dependent manner.

4) Mean rates of sulfated glycosaminoglycans synthesis by cultured equine SDFT explants as determined by sulfate incorporation were significantly increased in the presence of exogenous PSGAG at concentrations from 500 to 5000 μg/ml in a dose-dependent manner.

5) The mean rate of hyaluronate synthesis by cultured equine SDFT explants as determined by radioimmunoassay of the culture medium was significantly decreased in the presence of exogenous PSGAG at a concentration of 1000 μg/ml.

6) The mean rate of cell proliferation by cultured equine SDFT explants as determined by thymidine incorporation was significantly increased in the presence of exogenous PSGAG at a concentration of 1000 μg/ml.

9.8.2 Sodium Hyaluronate (Hyonate®)

1) Mean rates of protein synthesis by cultured equine SDFT explants as determined by proline incorporation were significantly increased in the presence of exogenous NaHA at concentrations between 500 to 2000 μg/ml in a dose-dependent manner.

2) Mean rates of collagen synthesis by cultured equine SDFT explants were significantly increased in the presence of exogenous NaHA at concentrations between 1000 to 2000 μg/ml in a dose-dependent manner.

3) Mean rates of noncollagen protein synthesis by cultured equine SDFT explants were not significantly affected by the presence of exogenous NaHA at concentrations between 50 to 2000 μg/ml.

4) Mean rates of sulfated glycosaminoglycans synthesis by cultured equine SDFT explants as determined by sulfate incorporation were significantly increased in the presence of exogenous NaHA at concentrations between 500 to 2000 μg/ml in a dose-dependent manner.

5) The mean rate of cell proliferation by cultured equine SDFT explants as determined by thymidine incorporation was not significantly increased in the presence of exogenous NaHA.

9.8.3 Insulin-like Growth Factor I

1) Mean rates of protein synthesis by cultured equine SDFT explants from a two-year-old horse as determined by proline incorporation were significantly increased in the presence of exogenous rhIGF-I at concentrations between 5 and 100 ng/ml in a dose dependent manner, but mean rates of protein synthesis by equine SDFT explants from a three and one half-year-old horse were not significantly increased in the presence of exogenous rhIGF-I at concentrations between 50 and 500 ng/ml.

2) Mean rates of collagen synthesis by cultured equine SDFT explants from a two-year-old horse were significantly increased in the presence of exogenous rhIGF-I at concentrations between 5 and 100 ng/ml in a dose-dependent manner, but mean rates of collagen synthesis by equine SDFT explants from a three and one half-year-old horse were significantly increased in the presence of exogenous rhIGF-I at 500 ng/ml only.

3) Mean rates of noncollagen protein synthesis by cultured equine SDFT explants from a two-year-old horse were significantly increased in the presence of exogenous rhIGF-I at concentrations between 5 and 100 ng/ml in a dose-dependent manner, but mean rates of noncollagen protein synthesis by equine SDFT explants from a three and one half-year-old horse were not significantly increased in the presence of exogenous rhIGF-I at concentrations between 50 and 500 ng/ml.

4) Mean rates of sulfated glycosaminoglycans synthesis by cultured equine SDFT explants from a two-year-old horse as determined by sulfate incorporation were significantly increased in the presence of exogenous rhIGF-I at concentrations between 5 and 100 ng/ml in a dose-dependent manner, but mean rates of sulfated glycosaminoglycan synthesis by equine SDFT explants from a three and one half-year-old horse were not significantly increased in the presence of exogenous rhIGF-I at concentrations between 50 and 500 ng/ml.

5) The mean rates of cell proliferation by cultured equine SDFT explants from a two-year-old horse as determined by thymidine incorporation were significantly increased in the presence of exogenous rhIGF-I at concentrations between 5 and 100 ng/ml in a dose-dependent manner.

CHAPTER TEN

10.0 BIBLIOGRAPHY

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CHAPTER ELEVEN

11.0 APPENDICES

11.1 Appendix I

Table A1: Signalment and source of experimental animals

Experiment	Animal	Source
Experiment 1:	14 mo female Quarter horse	Donation
Experiment 2:	3 yo male Quarter horse	Saskatoon Auction Mart
Experiment 3:	2 yo male Quarter horse cross-bred	Saskatoon Auction Mart
Experiment 4:	3.5 yo female Thoroughbred cross-bred	Saskatoon Auction Mart
Experiment 5:	2 yo male Quarter horse cross-bred	Saskatoon Auction Mart
Experiment 6:	2 yo female Thoroughbred	Privately owned farm

11.2 Appendix II - Materials and Equipment

Table A2: Source of essential materials and equipment

Product/Equipment	Manufacturer/Supplier
Tissue Culture	
Lactated Ringers 1 liter	Abbott Laboratories, QC, Canada.
Gentocin, Gentamicin sulfate 50 mg/ml	Schering Plough Animal Health, Shering Canada Inc., Pointe Claire, QC, Canada.
Fungizone, amphotericin B 200 μg/ml	Gibco BRL, Grand Island, NY, USA.
Penicillin G sodium 100, 000 IU	Novopharm Ltd, Toronto, ONT, Canada.
Acu-Punch® biopsy punch 4mm	Acuderm Inc., Fort Lauderdale, FL , USA.
20 mm Wheaton Aluminum seals	VWR Canlab, Edmonton, AB., Canada
30 ml Wheaton culture bottles	VWR Canlab, Edmonton, AB., Canada
20 mm rubber flange stopper	VWR Canlab, Edmonton, AB, Canada
Donor Horse Serum 100 ml	Gibco BRL, Grand Island, NY, USA.
RPMI Medium 1640 powder	Gibco BRL, Grand Island, NY, USA.
L-Ascorbic acid	Sigma Chemical Company, St.Louis, MO, USA.
Corning® Disposable Sterile Bottle Top Filters	Corning Laboratory Sciences, ONT, Canada.
Hyonate®hyaluronate sodium injection 20mg/ml	Bayer - Canada, Agricultural Products Division, Guelph, ONT, Canada
Adequan®IM polysulfated glycosaminoglycan	Luitpold Pharmaceuticals, Animal Health Division, Shirley, NY, USA.
Human recombinant IGF-I (CGP 35126)	Ciba Geigy, Animal Health Division, Mississauga, ONT, Canada
Human recombinant IGF-I (#01-141)	Upstate Biotechnology Incorporated, Lake Placid, NY, USA.

Table A2: Continued

Product/Equipment	Manufacturer/Supplier
Radiochemicals and Equipment	
Proline L-[2,3,4,5^{3}H]- 114.0 Ci/mmol, 5 mCi	Mandel Scientific, Geulph, ONT, Canada.
Sulfur-35 in water, carrier free 5.00 mCi/ml	Mandel Scientific, Geulph, ONT, Canada.
Methyl-^{14}C-thymidine	Amersham Life Sciences, Buckinghamshire,UK
Pharmacia HA Test (I^{125}HABP)	Pharmacia AB, Uppsala, Sweden.
BCS biodegradeable counting scintillant	Amersham Canada Ltd, Oakville ONT, Canada
Extran 300 Concentate BDH	VWR Canlab, Edmonton, AB, Canada
Beckman LS6000IC Liquid Scintillation System	Beckman Instruments, Mississauga, ONT, Canada
Minax 18 Autogamma 5000 series Gamma counter	United Technologies, Packard Instrument Company, Meriden, CT, USA.
RP-HPLC Equipment	
Alltech Absorbosphere OPA HR 5 μ, 150 x 4.6 mm, C fitting, HPLC column	Mandel Scientific., Geulph, ONT., Canada.
Alltech Absorbosphere OPA HR 5 μ, 7.5 x 4.6 mm, all guard cartridge column guard	Mandel Scientific., Geulph, ONT, Canada.
Alltech All-guard™ Guard cartridge holder	Mandel Scientific, Geulph, ONT, Canada.
Waters Data Module Model M7302 Waters M-45 Solvent Delivery System Waters M600A Solvent Delivery System	Millipore (Canada) Ltd, Waters Chromatography Division, Mississauga, ONT, Canada.

Table A2: Continued

Product/Equipment	Manufacturer/Supplier
Waters Model 720 System Controller Waters Model420 Fluorescence Detector Waters Model 450 Absorbance Detector	Millipore (Canada) Ltd, Waters Chromatography Division, Mississauga, ONT, Canada.
Rheodyne Syringe Loading Sample Injector Model 7125	Rheodyne Inc., Cotati, CA, USA.
Other Materials	
Solvable® tissue solubilizer	DuPont/NEN, Nbillerica, MA, USA.
Pro-X-Micro-Spin™	Lida Manufacturing Corp., WI, USA.
Bio-Spin® 6 Chromatography Columns	Bio-Rad Laboratories, Hercules, CA, USA.
VWR clear graduated pipette tips 200 µl	VWR Canlab, Edmonton, AB, Canada
VWR serological pipettes 5 ml	VWR Canlab, Edmonton, AB, Canada
Eppindorf ultra-micro tips 10 µl	VWR Canlab, Edmonton, AB, Canada
Cell Proliferation Kit RPN.20	Amersham Canada Ltd, ONT, Canada.

11.3 Appendix III

Laboratory Procedures Manual

1.0 Preparation Of Equine SDFT Explant Organ Cultures

1.1 Sterile removal of tendons from the donor horse

Materials

Sterile stainless steel tray and lid (20 cm x 16 cm)

- 1 litre Ringer's lactate
- high concentration sucrose solution
- 2 ug/ml Fungizone (2 mg)
- 100 ug/ml Gentamicin (100 mg)
- 100 IU/ml Crystalline penicillin (100,000 IU)

Euthanasia solution - pentobarbitone

Clippers

Chlorhexidine scrub

70% Alcohol

Sterile swabs/scrub brush

2 x pair sterile surgical gloves

4 x 2 sterile draping towels

2 x 4 Bachaus towel clamps

Sterile laceration pack with 3-4 number 22 scalpel blades

Protocol for removal of tendons from donor horse

1. Assemble all equipment required in the preparation area adjacent to the yellow recovery stall.

2. Euthanise/anaesthetize the horse in the yellow recovery room.

3. Suspend the forelimbs using the hoist and 1" white tape so that they are at a reasonable

working height and offset relative to each other.

4. Clip each forelimb from the accessory carpal bone to the bulbs of the heels over the palmar aspect for 270^0-360^0.

5. Surgically scrub the upper limb with chlorhexidine followed by an alcohol rinse. Repeat this procedure 3 times over the space of 5 minutes.

6. Place the saline and antimicrobials into the sterile stainless steel tray.

7. Open the laceration pack, towel clamps, scalpel blades and dressing towels in a sterile manner.

8. Put on sterile surgical gloves and '4-way' drape the leg leaving the prepared palmar aspect over the SDFT exposed.

9. Incise the skin in the midline over the SDFT from the accessory carpal bone to the ergot of the fetlock.

10. Sharply dissect away any peritendinous tissue and bluntly dissect between the SDFT and DDFT medially and laterally as far proximal as possible and distally to the tendon sheath.

11. Transect the tendon at the junction of the digital tendon sheath and as far proximal as possible, and place into the sterile saline containing antimicrobials.

12. Place the lid on top of the tray.

13. Repeat steps 5 to 12 for the other forelimb.

14. Place the tendons in their sterile tray into a tissue culture incubator at 37^0C until ready to dissect into segments.

1.2 Preparation of explant tissue segments from SDFT of donor horse

Materials

2 x pair sterile surgical gloves
Sterile laceration pack with 6 number 22 scalpel blades
10-20 x 4 mm biopsy punches
Gas sterilized plastic cutting board
Gas sterilized clamp apparatus (jig)
Sterile fume hood
RPMI 1640 culture medium
1-2 x 6-well culture plates

Protocol for dissection of tendons from donor horse

1. Open all equipment, including the tray containing the tendons, in a sterile manner in the fume hood and put on gloves.
2. Gently clamp the tendon perpendicularly in the jig on the cutting board and make transverse incisions in the tendon at 5 mm intervals using the plexiglass guide.
3. Remove the tendon slices and place into labeled culture wells according to their segmental location.
4. Using the biopsy punch and forceps, punch 3 to 4 explants from each slice.
5. Place the tray containing tendon segments in the incubator at 37^{0}C until the culture bottles containing media have been prepared.

1.3 Preparation of tissue culture media

Materials

Equipment & materials	Chemicals
1000 or 2000 ml beaker	Appropriate powdered media (RPMI 1640)
1000 ml measuring cylinder	1.5M sterile sodium bicarbonate solution 100mg/ml
Magnetic stirrer & bar	Sterile double distilled de-ionized water
pH meter & standards	10M NaOH
Electronic scale	1N HCl
Weigh trays	70% ethanol
Stainless steel spatula	20 mg/ml ascorbate solution

Sterile tissue culture washed 1000 ml Pyrex bottle(s) & orange cap(s)

500 ml funnel Corning disposable sterile bottle top filter (0.22 um pore size)

Water vacuum system	Electric pipette vacuum pump
Laminar flow hood	Sterile 2ml Pasteur pipettes
Gas sterilizing flame	Sterile 10 ml pipettes
CO_2 bottle	Drugs/additives of interest to be tested

Protocol for preparation of powdered culture media.

1. Calibrate the pH meter with the ph = 7 and pH = 10 standards.

2. Place a magnetic stirrer bar in a 1000 ml beaker on the stirrer base.

3. Measure 1000 ml of sterile double distilled de-ionized water into a measuring cylinder

4. Pour approximately 80 % of the water into the beaker and turn on the stirrer.

5. Shake the powdered medium down into the bottom of the packet and cut the top off.

6. Empty the packet of medium into the swirling water and wash the packet out into the beaker with the remaining water.

7. When the medium has dissolved into the water, add 20 ml/litre of medium of 1.5 M of sodium bicarbonate solution to the medium.

8. Measure the pH of the solution and adjust the pH of the media to 7.10±0.1 with 10M NaOH if it is too acidic, of 1N HCl if it is too basic.

9. Lift the fume hood to activate the laminar flow and wipe out the surfaces with 70% ethanol.

10. Place the media, a sterile tissue culture washed 1000 ml Pyrex bottle and sterile orange cap, a packaged 500 ml funnel Corning disposable sterile bottle top filter and suction line on the work surface within the laminar flow hood.

11. Carefully remove the foil from the top of the sterile Pyrex bottle maintaining sterility .

12. Carefully remove the filter unit from its packaging and without touching the threaded portion of the filter, screw the filter onto the sterile bottle.

13. Remove the dust cover from the filter and carefully pour the medium into the funnel.

14. Apply vacuum line to the filter (5-10 psi) and turn on the water vacuum.

15. Upon completion of filtration, disconnect the vacuum line prior to turning off the water to prevent water being sucked back into the filter unit.

16. Carefully remove the filter unit and flame the neck of the Pyrex bottle..

17. Remove the orange cap from its sterile packaging without touching the inside threaded portion and screw it onto the Pyrex bottle containing media.

18. Immediately label the medium with its type, the date, and your initials.

19. Cover the bottle with aluminium foil and refrigerate.

Protocol for preparation of serum containing media.

1. Into a sterile tissue culture washed Pyrex bottle of appropriate size, pipette the required volume of sterile serum free media with a sterile 10 ml pipette.
2. For each 100ml of medium, pipette the 10 ml of sterile filtered serum into the Pyrex bottle.
3. Pipette the required volume of ascorbate (to make a concentration of 50 µg/ml) into the medium. eg. For 100 mls RPMI 1640 media containing 10% DHS add 250 ul of 20 mg/ml ascorbate
4. Flame sterilize the neck of the Pyrex bottle and place a sterile orange cap on without touching the neck of the bottle, and mix the contents of the bottle by using a gentle swirling action.
5. Immediately label the media with its type, the date, and the initials of the person who prepared it.

N.B. If at any time a sterile piece of equipment comes into contact with a non-sterile surface (eg.hand), discard the equipment and get a new piece of sterile glassware. Change pipettes between different media.

1.4 Preparation of explant tissue cultures

Materials

Laminar flow hood
Sterile prepared media with serum and ascorbate
Sterile culture washed 30 ml Wheaton bottles (1 per tissue segment)
Tendon segments
Sterile thumb forceps
Sterile 10 ml pipettes
Electric pipette vacuum pump
Sterile plugs for Wheaton bottles
Aluminium seals for Wheaton bottles
Manual press for seals
Sterile 20 G needles
Gas cylinder containing 50% O_2 45% N_2 5% $C0_2$
Millepore filter (0.22 um)
70% ethanol
Roller modified incubator set at 36.5^{0}C

Protocol for preparation of cultures.

1. Lift the fume hood glass to activate the laminar flow and wipe all surfaces with 70% ethanol.
2. Place the cylinder of pipettes, the desired number of labeled sterile Wheaton bottles, the sterile plugs, sterile forceps and pipette vacuum handle, and media onto the work surface within the laminar flow hood.
3. Carefully remove the foil from the top of the sterile Wheaton bottles ensuring that the necks of the bottles are not touched.
4. Carefully remove the cap from the top of the sterile Pyrex bottle containing the media,

ensuring that the neck of the bottle is not touched.

5. Using the tips of the sterile thumb forceps, carefully transfer one tendon segment per Wheaton bottle from the sterile tray containing the dissected tendon.

6. With a sterile 10 ml pipette, transfer 4 ml of appropriate media into each Wheaton bottle.

7. Carefully place the sterile plugs on top of the bottles using sterile forceps, then gently seat the plugs into the neck of the Wheaton bottles using gently thumb pressure, taking care not to touch the rim or the neck of the bottles.

8. Place the aluminium caps over the plugs and seal them using the manual press.

9. Remove the centres of the seals by gentle pressure on the centres or using the wall mounted opener.

10. Sterilize the top of the bottles with alcohol.

11. Place the millepore filter on the outflow tubing from the gas cylinder.

12. Place a sterile 20G needle on the millepore filter.

13. Insert the needle into the exposed sterile portion of the plug, and insert another sterile 20 G needle for an outflow, taking care not to touch either the needle shafts or the exposed rubber plug.

14. Turn on the gas at 15 psi for 15-20 seconds.

15. Remove the needles without touching the shafts, and continue gassing the other bottles.

16. When all the cultures have been gassed, place them on the rollers, 5 per row, with the caps facing towards the incubator door.

17. Repeat the gassing procedures every 24 hours for all cultures.

18. Change the media every 72 hours.

N.B. If at any time a sterile piece of equipment comes into contact with a non-sterile surface (eg.hand), discard the equipment and get a new piece of sterile glassware. Change pipettes between different media.

1.5 Changing the medium of explant tissue cultures

Materials

Laminar flow hood
Sterile prepared medium with serum and ascorbate
Wheaton bottles containing tendon segments
Multigrips or vial opener for cap removal
Sterile 10 ml pipettes
Electric pipette vacuum pump
Sterile thumb forceps
Sterile plugs for Wheaton bottles
Aluminium seals for Wheaton bottles
Manual press for seals
Sterile 20 G needles
Gas cylinder containing 50% O_2 45% N_2 5% $C0_2$
Millepore filter (0.22 um)
70% ethanol
Betadine scrub
Roller modified incubator set at 37.5°C

Preparation of cultures.

1. Lift the fume hood glass to activate the laminar flow and wipe out the hood surfaces with 70% ethanol.
2. Place the cylinder of pipettes, the labeled Wheaton bottles containing the cultures, the sterile plugs, sterile forceps and pipette vacuum handle, and media onto the work surface within the laminar flow hood.
3. Carefully remove the caps and plugs Wheaton bottles ensuring that the necks of the bottles are not touched.

4. Using a fresh sterile pipette every 3-4 cultures (1 per culture if problems with contamination have been experienced), remove and discard 50% (2ml) of the used media.

5. Carefully remove the cap from the top of the sterile Pyrex bottle containing the media, ensuring that the neck of the bottle is not touched.

6. With a sterile 10 ml pipette, transfer 2 ml of appropriate fresh medium into each Wheaton bottle.

7. Carefully place the sterile plugs on top of the bottles using sterile forceps, then gently seat the plugs into the neck of the Wheaton bottles using gently thumb pressure, taking care not to touch the rim or the neck of the bottles.

8. Place the aluminium caps over the plugs and seal them using the manual press.

9. Remove the centres of the seals by gentle pressure on the centres or using the wall mounted opener.

10. Sterilize the top of the bottles first with betadine scrub, then with alcohol.

11. Place the millepore filter on the outflow tubing from the gas cylinder.

12. Place a sterile 20G needle on the millepore filter.

13. Insert the needle into the exposed sterile portion of the plug, and insert another sterile 20 G needle for an outflow, taking care not to touch either the needle shafts or the exposed rubber plug.

14. Turn on the gas at 15 psi for 15-20 seconds.

15. Remove the needles without touching the shafts, and continue gassing the other bottles.

16. When all the cultures have been gassed, place them on the rollers, 5 per row, with the caps facing towards the incubator door.

17. Change the media every 72 hours.

N.B. If at any time a sterile piece of equipment comes into contact with a non-sterile surface (eg.hand), discard the equipment and get a new piece of sterile glassware. Change pipettes between different media.

1.6 Gassing of cultures

Materials

Sterile 20 G needles

Gas cylinder containing 50%O_2/45%N_2/5% $C0_2$

Millepore filter (0.22 um)

70% ethanol

Betadine scrub

Roller modified incubator set at 37.5^{0}C

Procedure for gassing cultures.

1. Sterilize the top of the bottles first with betadine scrub, then with alcohol.

2. Place the millepore filter on the outflow tubing from the gas cylinder.

3. Place a sterile 20G needle on the millepore filter.

4. Insert the needle into the exposed sterile portion of the plug, and insert another sterile 20 G needle for an outflow, taking care not to touch either the needle shafts or the exposed rubber plug.

5. Turn on the gas at 15 psi for 15-20 seconds.

6. Remove the needle without touching the shafts, and continue gassing the other bottles.

7. When all the cultures have been gassed, place them on the rollers, 5 per row, with the caps facing towards the incubator door.

8. Repeat the gassing procedures every 24 - 72 hours as required for all cultures.

N.B. If at any time a sterile piece of equipment comes into contact with a non-sterile surface (eg.hand), discard the equipment and get a new piece of sterile equipment.

2.0 Radiolabeling Procedures

2.1 Radiolabeling of equine superficial digital flexor tendon explant organ cultures

Materials

Laminar flow hood
Sterile prepared media without serum
Ascorbate 20 mg/ml
Proline 20 mg/ml
Wheaton bottles containing tendon segments
Multigrips for cap removal
Sterile 10 ml pipettes
Sterile disposable 5 ml pipettes
Plastic 10 ml capped vials
Glass 3 ml vials
Electric pipette vacuum pump
10 ul Pipette and sterile tips
Tritiated proline (1000 μCi/ml) and labeled sulphate (1050-1600μCi/ml)
Radioactive waste disposal containers
Disposable plastic backed bench protector
Gloves
Sterile thumb forceps
Sterile plugs and aluminum seals for Wheaton bottles
Manual press for seals
Sterile 20 G needles
Gas cylinder containing 50% O_2 45% N_2 5% $C0_2$
Millepore filter (0.22 um)

N.B. Wear gloves and protective clothing for all procedures associated with the handling and disposal of radioactive materials.

Protocol for radiolabeling of SDFT cultures.

1. Lift the fume hood glass to activate the laminar flow and wipe out the hood surfaces with 70% ethanol.
2. Place sufficient bench protector on the work surface within the laminar flow hood for all the Wheaton bottles and the isotope container.
3. Place the cylinder of pipettes, the sterile plugs, sterile forceps and pipette vacuum handle, the micropipettor and sterile tips onto the work surface within the laminar flow hood.
4. Place the Wheaton bottles containing the cultures on the bench protector.
5. Carefully remove the caps and plugs Wheaton bottles with the multigrips ensuring that the necks of the bottles are not touched.
6. Using a fresh sterile Pasteur pipette every 3-4 cultures (1 per culture if problems with contamination have been experienced), aspirate the used media with the water vacuum into a volumetric flask and discard or keep for later use.
7. Carefully remove the cap from the top of the sterile Pyrex bottle containing the media, ensuring that the neck of the bottle is not touched.
8. With a sterile 10 ml pipette, transfer 4 ml of and serum free RPMI 1640 media into each Wheaton bottle and aerate with 5%CO_2.
9. Wearing gloves and glasses, remove the isotope containers from the refrigerator, place it in the fume hood on the bench protector, and carefully remove the caps from the containers.
10. Using a new sterile tip each time, transfer 20µl (20µCi/culture) of radiolabeled proline and (25 µCi/culture) radiolabeled sulphate to each culture, ensuring the tip of the pipette touches the inside of the neck of the Wheaton bottle to ensure accurate transfer.
11. Discard each tip into a radioactive waste container.
12. Carefully place the sterile plugs on top of the bottles using sterile forceps, then gently seat the plugs into the neck of the Wheaton bottles using gently thumb pressure, taking care not to touch the rim or the neck of the bottles.
13. Place the aluminium caps over the plugs and seal them using the manual press.
14. Remove the centres of the seals by gentle pressure on the centres.
15. Place cultures on the rollers, 5 per row, with the caps facing towards the incubator door.

Protocol for chase incubation of radiolabeled of SDFT cultures.

1. Lift the fume hood glass to activate the laminar flow and wipe out the hood surfaces with 70% ethanol.

2. Place the sufficient bench protector on the work surface within the laminar flow hood for all the Wheaton bottles and the isotope container.

3. Place the cylinder of pipettes, the sterile plugs, sterile forceps and pipette vacuum handle, the micropipettor and sterile tips onto the work surface within the laminar flow hood.

4. Wearing gloves, place the Wheaton bottles containing the radiolabeled cultures on the bench protector.

5. Carefully remove the caps and plugs from the Wheaton bottles with the multigrips ensuring that the necks of the bottles are not touched, and discard the caps into a radioactive waste container.

6. Using a fresh sterile pipette for every culture, remove the radioactive media from each bottle and place into individually labeled plastic 10 ml capped vials.

7. Carefully remove the cap from the top of the sterile Pyrex bottle containing the serum free media, ensuring that the neck of the bottle is not touched.

8. With a sterile 10 ml pipette, transfer 2 or 4 ml of appropriate serum free media with added proline into each Wheaton bottle and aerate with 5%CO_2.

9. Carefully place the sterile plugs on top of the bottles using sterile forceps, then gently seat the plugs into the neck of the Wheaton bottles using gently thumb pressure, taking care not to touch the rim or the neck of the bottles.

10. Place the aluminium caps over the plugs and seal them using the manual press.

11. Place the cultures on the rollers, 5 per row, with the caps facing towards the incubator door for 24 hours.

12. Harvest all chase media and tendon segments 24 hours after placement in incubator, placing media samples into labeled plastic 10 ml tubes and tissues into plastic 7 ml scintillation vials.

13. Store all samples in the -70°C freezer for later analysis.

3.0 Preparative Procedures and Analysis of Radiolabeled Tissue Samples

3.1 Hydrolysis and Scintillation Counting of Radiolabeled Tissue Samples

Materials

Radiolabeled tissue

6N HCl

2ml glass lyophilization vials

Water vacuum

Bunsen burner

N_2 gas

Hot water bath

Solvable® tissue solubilizer

Hydrogen peroxide

7 ml Polyethylene scintillation vials

BCS scintillation fluid

Protocol

1. Freeze dry tendon radiolabeled tendon segments.
2. Weigh freeze dried tendon segments.
3. Placed each freeze dried explant in a labeled 2ml lyophilization tube.
4. Add 1 ml of warm 6 M HCl and apply the tubing connected to the water vacuum to the mouth of the vial.
5. While the vacuum is applied, heat the neck of the lyophilization tube until the glass is plastic, and draw out the glass until a seal is created, then using a circular motion, remove the glass at the open end and discard.

6. Hydrolyse the tendon segments in 1 ml of 6M HCl at 110 °C for 24 hours.

7. Remove the hydrolysates from the heat and allow to cool. before

8. Vortex gently, then transfer 500 µl of aliquot of the hydrolysate to a labeled scintillation vial and dry sample with nitrogen.

9. Transfer the remainder of the hydrolysate to 1.5 ml microfuge vials with 0.22 µm pore size microfuge filter and centrifuge at 3000 rpm for 5 minutes. Conserve and vacuum centrifuge filtrate and then freeze at -20°C for later derivatization.(Vacuum centrifuge in Veterinary Microbiology)

10. To the freeze dried samples in scintillation vials add tissue solubilizer as per guideline below.

11. Cocktail addition: Add 6 ml of cocktail to each vial. (Samples > 150 mg may need up to 15 ml to clear).

12. Count in scintillation counter on 2 channels (^{3}H and ^{35}S) with color quench correction, using the customized quench curve installed by Riley.(Counter in Dow Elanco laboratory, Animal Science)

Tissue solubilization for samples up to 300mg (3hrs)

I. Add solvable to the minced fresh tissue sample in a glass scintillation vial -

< 50 mg add 0.5 ml; < 200 mg add 1.0 ml; < 300 mg add 1.5 ml

NB. Tissue sample must be completely immersed. Gently agitate the sample; do not vortex because pieces of the tissue will adhere to the walls of the scintillation vials.

II. Incubate at 50 °C until clear - approximately 3 hours.

III. Decolorization (optional): hydrogen peroxide is the best bleaching agent, but foaming may be a problem. Add 0.1-0.2 ml 30% H_2O_2 to each vial and incubate at room temperature for one hour.

NB. If > 0.2 ml of hydrogen peroxide is needed, add in aliquots to prevent foaming.

3.2 Pre-column derivatization of amino acids in tendon digest with dabsyl chloride

Materials

Aliquot of tendon hydrolysate

Dabsyl chloride (Sigma)

100mM carbonate-bicarbonate buffer solution (pH 8.30) HPLC grade

7.5 M KOH HPLC grade

Acetone HPLC grade

Acetonitrile HPLC grade

Standard amino acids mixture

L-Proline standard (*mw* 115.1)

OH-L-Proline standard (*mw* 131.1)

Norleucine standard

Distilled water

Glass 10/20 ml scintillation vials

Syringe filters

20 ml syringe

18G needle

Protocol

Dabsyl chloride preparation

1. Rinse 2 scintillation vials with acetonitrile.
2. Dissolve 13 mg of dabsyl chloride in 10 mls acetonitrile in a vial.
3. Vortex to dissolve - heat if necessary.
4. Syringe filter into another vial (good for 2 weeks, but better to make up each day).

Derivatization

1. Prepare a solutions of standard amino acids as required:

OH-L-Proline	10 µM/ml
L-Proline	10 µM/ml
Norleucine	10 µM/ml
Glycine	10 µM/ml
Standard amino acid mix	2.5 µM/ml

Combinations/dilutions of the above in buffer

2. Adjust pH of each hydrolysate to 9.0 ± 0.2 with KOH and measure total volume.

3. Add 40 µl of 100 mM sodium bicarbonate (pH 8.3) to microfuge tube.

4. Add 40 µl of sample or standard to the tube (may add 5 µl of norleucine as internal standard if desired).

5. Add 80 µl of Dbs-Cl to tube, cover with paraffin film and incubate for 12 minutes at 70°C.

6. Vortex at time = 1 minute.

7. Vortex at time = 4 minutes.

8. Remove from heat at 12 minutes and allow to cool for 5 minutes.

9. Add 440 µl of 100 mM sodium bicarbonate, seal and gently invert.

10. Aspirate 20 µl samples into a Hamilton syringe, expel any air and wipe the needle clean, and inject into the RP-HPLC column.

4.0 Preparative Procedures and Analysis of Radiolabeled Media Samples

4.1 Preparation of radiolabeled medium for scintillation counting

Materials

Radiolabeled medium
Bio-Spin®6 chromatography columns (732-6002)
7 ml Polyethylene scintillation vials
Solvable® tissue solubilizer
BCS scintillation fluid

Protocol

1. Invert each column several times to resuspend settled gel.
2. Remove the top cap and then snap off the snap-off tip to allow excess buffer to drain by gravity (discard buffer).
3. Place the column in a collection tube and centrifuge at 1,100 x g (2300 rpm) for 2 minutes in a swinging bucket centrifuge.
4. Re-spin if any buffer remains in the tip of the column. Store the buffer and collection tube.
5. Carefully apply 100 µl of radiolabeled medium to the centre of the column, allowing the liquid to drain into the gel bed between successive drops of the sample.
NB. Avoid applying the sample to the sides of the column and avoid adding more than the recommended amount.
6. Place the column containing the applied sample in a clean collection tube and centrifuge for 4 minutes at 1,100 x g.
7. Discard the column into the radioactive waste or recycle using SCC buffer and transfer the eluent to a scintillation vial.
8. Solubilize with 0.5 ml of solvable for 3 hours at 50°C, then add 6 ml of scintillant.
9. Count in scintillation counter on 2 channels (^{3}H and ^{35}S) with color quench correction.

4.2 Preparation of protein in radiolabeled culture medium for pre-column derivatization

Materials

Radiolabeled medium
Standard amino acids with norleucine
Distilled water
Microfuge tubes
0.15% DOCA (7-Deoxycholic acid)
72% TCA (Trichloracetic acid)
6 N HCl

Protocol

1. Transfer 1 ml of radiolabeled medium to a 1.5 ml microfuge tube (may make duplicates if required).
2. A 100 µl of 0.15% DOCA to culture medium, mix and allow to stand for 10 minutes at room temperature (20-25^{0}C).
3. Add 100 µl of 72% TCA to each tube, mix and centrifuge at 3300g for 30 minutes.
4. Decant or aspirate supernatant and dry precipitate from samples in vacuum microfuge.
5. Freeze at precipitant at -70^{0}C for storage, or continue with protocol.
6. Add 100 µl (or 10 x the weight of protein, whichever is the greater) of 6 N HCl to each microfuge tube, displace the air with nitrogen gas, seal tube and incubate at 110^{0}C for 24 hours.
7. Vacuum centrifuge or dry with nitrogen gas to remove HCl and derivatize amino acids.

4.3 Pre-column derivatization of protein hydrolysate from radiolabeled culture medium with dabsyl chloride

Materials

Radiolabeled medium

Dabsyl chloride

100mM carbonate-bicarbonate buffer solution (pH 8.3)

Glass 10/20 ml scintillation vials

Syringe filters

20 ml syringe

18G needle

Acetone

Acetonitrile

Standard amino acids

Norleucine

Distilled water

Protocol

Dabsyl chloride preparation

1. Rinse 2 scintillation vials with acetonitrile.
2. Dissolve 13 mg of dabsyl chloride in 10 mls acetonitrile in a vial.
3. Vortex to dissolve - heat if necessary.
4. Syringe filter into another vial (good for 2 weeks, but better to make up each day).

Derivatization

1. Prepare a solutions of standard amino acids as required:

OH-L-Proline	10 µM/ml
L-Proline	10 µM/ml
Norleucine	10 µM/ml
Glycine	10 µM/ml
Standard amino acid mix	2.5 µM/ml

Combinations/dilutions of the above in buffer

2. Adjust pH of each hydrolysate to 9.0 ± 0.2 with KOH and measure total volume.

3. Add 40 µl of 100 mM sodium bicarbonate (pH 8.3) to microfuge tube.

4. Add 40 µl of sample or standard to the tube (may add 5 µl of norleucine as internal standard).

5. Add 80 µl of Dbs-Cl to tube, cover with paraffin film and incubate for 12 minutes at 70°C.

6. Vortex at time = 1 minute.

7. Vortex at time = 4 minutes.

8. Remove from heat at 12 minutes and allow to cool for 5 minutes.

9. Add 440 µl of 100 mM sodium bicarbonate, seal and gently invert.

10. Aspirate 20 µl samples into a Hamilton syringe, expel any air and wipe the needle clean, and inject into the RP-HPLC column.

5.0 Reverse Phase High Pressure Liquid Chromatography

Materials

Waters HPLC apparatus

Millepore® Waters Data Module M730
Millepore® Waters System Controller M720
Millepore® Waters Solvent Delivery System M45 (B)
Millepore® Waters Solvent Delivery System M6000A (A)
Waters Lambda Max LC UV Absorbance Detector M481 (variable □ set at 436 nm)
Rheodyne Syringe Loading Sample Injector M7125

Alltech Adsorbosphere OPA-HS 5 µm (100 x 4.6 mm) column
Alltech Adsorbosphere guard column and cartridges
Column heater at 30°C (custom made)
Hamilton syringe 100 µl
Fraction collector

Solvent A:

56% 100 mM Sodium acetate (5.8 ml Glacial acetic acid/litre) }
16% Acetonitrile }
28% Methanol } pH=5.8 v/v/v
0 to 0.5% Tetrahydrafurans }

Solvent B:

100% Methanol

Nylon filter and Millipore filter apparatus

7 ml Polyethylene scintillation vials

BCS scintillation fluid

All chemical used should be HPLC grade or better

Protocol

Solvent Preparation

1. Prepare solvent A 100 mM sodium acetate by adding 5.8 ml of glacial acetic acid per 1000 ml of double distilled water and adjust pH to 5.8.

2. Add the remaining components to solvent A as formulated above, adjust pH to 5.8, filter and degass.

3. Prepare solvent B, filter and degass.

Operation of RP-HPLC Apparatus

1. Turn on column heater at least 12 hours before running samples/

2. Turn on controller, pumps, module, detector (0.1 AUFS; 436 nm) and fraction collector.

3. Program controller with gradient.

4. Run 100% ethanol through both pumps for 30 minutes each at 1.0ml/min and check flow rates.

5. Check UV monitor for evidence of gas bubbles and bleed both pumps carefully.

6. Calculate compressibility coefficients for each solvent and calibrate the pumps using the controller.

$$cf = \left(\frac{Time}{Volume/Flow\ rate} - 1 \right) \times 100 \times \frac{6000}{Pressure}$$

7. Check flow rates again and adjust as necessary.

8. Check column temperature has stabilized at 32^{0}C.

9. Set fraction collector to peak collection (10% threshold).

10. Apply 20 µl of a standard sample to column with Hamilton syringe.

11. Initiate preset chromatography program and check the chromatogram.

12. Fine tune the system as necessary before applying next sample.

13. Run standards.

14. Run test samples.

15. Collect 0.5 or 1.0 ml fractions from the outflow tubing of the absorbance detector into 7 ml scintillation vials.

16. Add 6 ml of BCS scintillant, cap and mix gently.

17. Count in scintillation counter on 2 channels (^{3}H and ^{35}S) with quench correction.

Isocratic gradient.

Time	Flow	Solvent		Gradient
(mins)	(ml/min)	A(%)	B(%)	
Initial	1	100	0	*
5	1	100	0	6
10	1	99	1	6
15	1	65	35	6
18	1	60	40	6
20	1	0	100	6
24	1	0	100	6
26	1	100	0	6

6.0 Identification of Cell Proliferation In Vitro

Materials

Amersham cell proliferation kit

1. Labeling reagent (5-bromo-2'-deoxyuridine & 5fluoro-2'-deoxyuridine in 10:1 ratio)
2. Nuclease
3. Anti-bromo-deoxyuridine monoclonal IgG2A antibody (murine)
4. Peroxidase anti-mouse IgG2a (concentrate)
5. Peroxidase anti-mouse IgG2a diluent
6. Substrate intensifier - hydrogen peroxide with cobalt chloride and nickel chloride
7. DAB - 3,3'-diaminobenzidine tetrahydrochloride

10% Sheep serum

Humidified chamber

Coplin jar

Tissues

RPMI 1640 medium

Xylene

70, 95 and 100% ethanol

Phosphate buffered saline

Phosphate buffer

Harris hematoxylin

Eosin

NB. See kit guideline for notes on preparation and storage of saline, buffer and reagents.

Protocols

6.1 In vitro labelling with 5-bromo-2'-deoxyuridine (BrdU)

1. Prepare labeling medium by diluting reagent 1:1000 with complete tissue culture medium and sterilize by filtration through a 0.22 μm filter. Warm to 37°C before use.
2. Add 10 ml of warmed labeling medium to the tissues in culture vessels.
3. Add 100 μl of 30%(v/v) hydrogen peroxide and seal culture vessel. Discard unused labeling medium.
4. Incubate at 37°C for 24 hours in incubator.
5. Wash tissue in PBS for 15 minutes at 37°C.
6. Fix tissue in 70% ethanol and embed in paraffin as required. Paraffin wax temperature must not exceed 58°C (see kit guidelines for detailed protocol).
7. Cut 3-5 μm sections from the outer 100 μm of the tissue exposed to the labeling medium.

6.2 De-waxing and rehydration of paraffin embedded tissue sections

1. Immerse sections in xylene for 2-3 minutes (Do not let sections dry out between steps).
2. Repeat with 2 changes of fresh xylene (replace solvents regularly, ie every 40 slides).
3. Immerse sections in 100% ethanol for 2-3 minutes.
4. Repeat with 1 change of fresh 100% ethanol.
5. Immerse sections in 70% ethanol for 2-3 minutes.
6. Immerse sections in aqueous (phosphate) buffer for 2-3 minutes.

6.3 Blocking of endogenous peroxidase

1. Immerse sections in 0.3% H_2O_2 in methanol for 30 minutes at room temperature.
2. Wash 3x with phosphate buffer.

6.4 Immunocytochemistry protocol

1. De-wax paraffin sections and inactivate endogenous peroxidase.
2. Rehydrate specimen in washing 3 x in PBS, drain and wipe away excess solution around specimen.
3. Add sufficient 10% sheep serum to cover specimen and place in humidifying chamber for 20 minutes.
4. Drain sheep serum off (do not rinse).
5. Add sufficient reconstituted nuclease/anti-BrdU to cover specimen.
6. Incubate for 1 hour at room temperature in humidifed chamber.
7. Wash 3 x with PBS (3 minutes each time minimum). Wipe around specimen.
8. Add sufficient peroxidase anti-mouse IgG2a to cover specimen.
9. Incubate for 30 minutes at room temperature.
10. During this incubation period thaw out one aliquot of DAB concentrate and dilute into 50 ml of phosphate buffer.
11. Wash slides with 3 x PBS (3 minutes each time minimum).
12. Add substrate/intensifier to dilute DAB solution. Use 5 drops/ DAB, stir vigorously during addition of drops.
13. Immerse slides in DAB staining solution for 5-10 minutes.
14. Wash slides in 3 x distilled water.
15 Counterstain as desired - weak nuclear stain (eg.Harris hematoxylin) or cytoplasmic stain is recommended.

6.5 Counterstaining

1. Stain in Harris hematoxylin for 20 seconds (less for a weaker staining reaction).
2. Wash well in 3 rinses of distilled water.
3. Quickly dip (1 second) in 0.5% acid alcohol.
4. Blue in sodium diphosphate.
5. Wash well in 3 rinses of distilled water.

6. Immerse in 70% ethanol for 1 minute.

7. Immerse in 95% ethanol for 1 minute.

8. Stain for 20 seconds in 0.1% alcoholic eosin.

9. Immerse in 100% ethanol for 1 minute.

10. Repeat once in 100% ethanol for 1 minute.

11. Clear in 2 changes of xylene each for 2 minutes.

12. Mount in synthetic resin

7.0 Hyaluronate Radiometric Assay

Materials

Pharmacia HA Test Kits (I^{125}HABP)

100 µl micropipette with disposable tips

100 µl and 200 µl repeating pipettes

Test tubes

Centrifuge capable of 1500 x g

Gamma Counter

Protocol

1. Pipette 100 µl of HA standards (0,5, 10, 15, 25, 50, 75, 100, 200, 500 and 1000 µg/l) and unknown samples into tubes.

2. Add 200 µl of HABP-^{125}I solution to tubes including two extra tubes to determine the activity of the solution.

3. Incubate for at least 60 miutes at 4 to 20°C.

4. Add 100 µl of HA-sepharose after thoroughly resuspending the beads.

5. Incubate for 45 minutes but not more than 60 minutes at 4 to 20°C.

6. Add 2 ml of decanting solution after shaking it before use.

7. Centrifuge for 10 minutes at 1500 x g, then decant tubes in one movement and allow to stand upside down on absorbent paper - the firmly packed precipitate should remain on the bottom of the tubes.

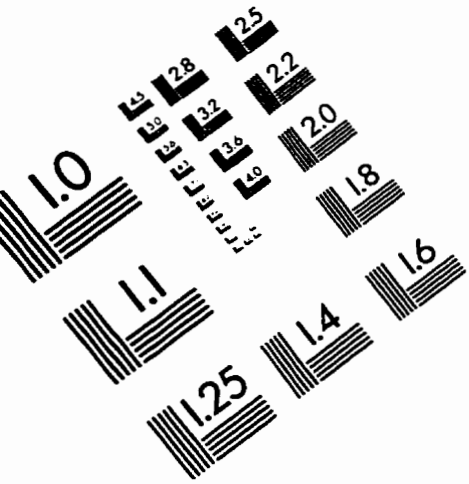
8. Measure the radioactivity in a gamma counter.

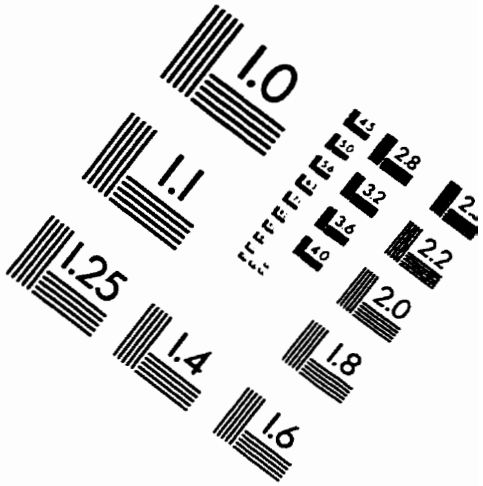
9. Express the counts (B) for the standards and unknowns as a percentage of the mean counts of the O-standard (B_0)

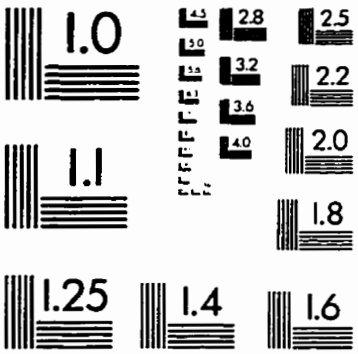
$$\% \text{ activity bound} = B/B_0 \times 100$$

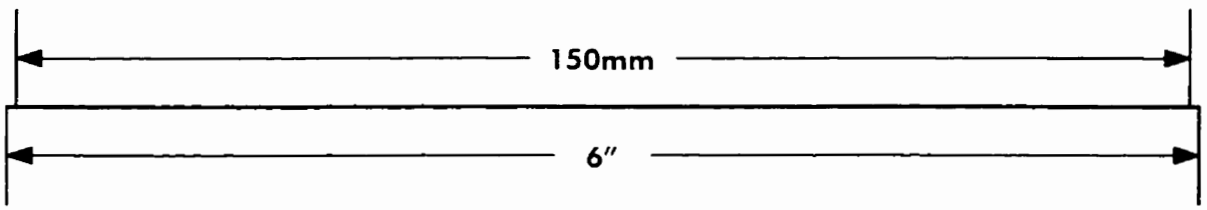
10. Plot the percentage values of the standards against the HA concentration on linear-log paper and construct a standard curve (alternatively can use curve fitting software to develop an equation describing the relationship) and read unknown samples from the curve.

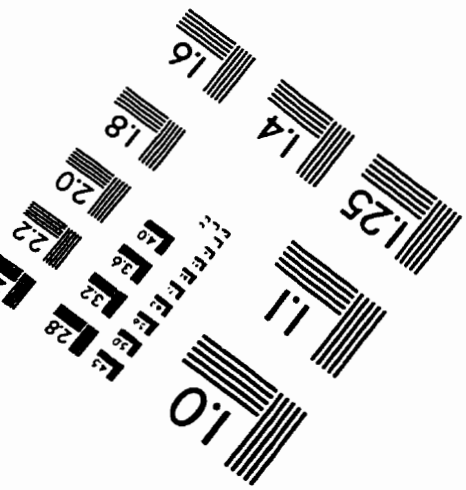
IMAGE EVALUATION TEST TARGET (QA-3)

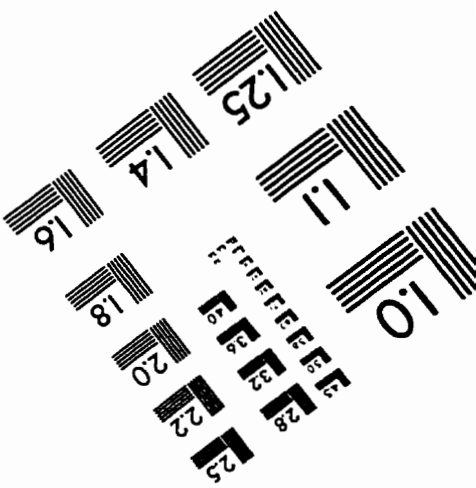












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